Remarks

Claims 8-11 and 14-16 are pending in the application. Reconsideration and allowance is requested in view of the above changes and the following remarks.

Claims 8, 9 and 16 have been amended to recite that the composition is capable of eliciting both a cytotoxic T-cell and antibody response immune response. Support for the introduction of this feature can be derived from the description at page 14, lines 8-17 and further at page 15, lines 20-27. Support for the introduction into the claims of the feature that the "stress protein complexes are not purified to homogeneity" can be derived from the description at page 13, lines 21-24, and page 10, lines 24-26.

Basis for the feature of the stress treatment stimulating "the presence of stress proteins within the infected cells", as included in amended claim 16, can be found at page 7, lines 7 to 9.

Basis for the stress protein of the stress protein complex being derived from the infected cell can be derived from throughout the specification, and in particular page 9, lines 4-11, and from the examples. Basis for the stress protein being derived from the intracellular pathogen can be derived from page 6, lines 28-30. Basis for the antigenic peptide fragment of the stress protein complex being derived from the intracellular pathogen can be derived from page 7, lines 16-21, and from throughout the specification and in particular the examples.

Response to Section 112 Rejection

Claims 8-11 and 14-16 have been rejected as being allegedly indefinite. Examiner alleges that the claims are drawn to compositions comprising a complex of any "induced stressed protein" and "any antigenic peptide fragment". Examiner alleges that the mere recitation of the name, "stress protein/antigenic peptide" in not sufficient to satisfy Section 112, 2nd to adequately define what is claimed, and that this name is used for a vast number of proteins allegedly present in the prior art. Examiner alleges that product-by process limitations present in the composition claims do not define structure; the claim should provide structural properties such as amino acid

sequence or molecular weight, to allow one to identify a particular protein (ostensibly the complex of the stress-induced stress protein and antigenic peptide fragment).

Claims 8 and 9 define a composition comprising an immunogenic determinant comprising complexes formed between a stress induced stress protein and an antigenic peptide fragment. Claim 16 defines a composition of the determinant comprising complexes between a stress induced stress protein and an antigenic peptide fragment according to its method of preparation. The claims do not recite, as Examiner alleges, the "name" "stress protein/antigenic peptide". The expression "stress protein/antigenic peptide" does not appear in the claims. Moreover, the claims do not merely reference complexes of stress proteins and antigenic peptides in the abstract, but characterize the complexes, or their constituent components, by functional properties and/or their methods of preparation.

The test of indefiniteness is whether one skilled in the art would understand what is claimed when the claim is read in light of the specification. *Orthokinetics Inc. v. Safety Travel Hairts, Inc.*, 1 USPQ2d 1081, 1088 (Fed. Cir. 1986); *Miles Labs Inc. v. Shandon Inc.*, 27 USPQ2d 1123, 1126 (Fed. Cir. 1993). The degree of precision necessary is a function of the subject matter claimed. *Hybritech Inc. v. Monoclonal Antibodies, Inc.* 231 USPQ 81 (Fed. Cir. 1986). The relevant inquiry in determining whether a given claim satisfies the requirements of 35 USC 112, second paragraph, is whether the claim sets out and circumscribes a particular area with a reasonable degree of precision and particularity. *In re Moore*, 169 USPQ 236, 238 (CCPA 1971). Indefiniteness of claim language may not be analyzed in the abstract, but must always be considered in light of the teachings of the prior art and of the particular application disclosure, as it would be interpreted by one of ordinary skill in the pertinent art. *Id.*

The claims distinctly point out and define that the complexes are formed between a stress-induced stress protein and an antigenic peptide fragment, and characterize the complex and its components according to origin or function. The claims recite certain features that the complexes are obtained from a cell which has been infected with a bacterial, protozoal or parasitic intracellular pathogen, which infected cell has been subject to stress with heat or tumor

necrosis factor sufficient to stimulate the presence of stress proteins within the infected cell. The claims further recite that the complexes are derived from either the infected cell or from the intracellular pathogen, and that the antigenic peptide fragment in eh complexes is derived from the intracellular pathogen. These features point out and distinctly claim the invention, and, for the reasons stated later in this paper, distinguish the invention over the prior art. Examiner has not explained *why* the skilled artisan would not understand these features of the claims when read in light of the specification, or *why* the skilled artisan would be unable to determine the boundaries of the subject matter for which protection is sought.

The absence of "structural properties" such as amino acid sequence or molecular weight is not determinative of whether the claims satisfy Section 112, 2nd paragraph. A claim may not be rejected solely because of the type of language used to define the subject matter for which patent protection is sought. *In re Swinehart*, 160 USPQ 226 (CCPA 1971). Applicant is free to define the invention in his own terms, including characterizing the composition and its components in terms of their properties or methods of preparation. As to the allegation that the "stress protein/antigenic peptide", apparently referring to the complexes of the invention, is used for a "vast number of different proteins", this is not a basis for rejection under Section 112, 2nd paragraph. Breadth of a claim is not to be equated with indefiniteness. *In re Miller*, 169 USPQ 597 (CCPA 1971). The absence of a limitation in a claim which broadens the claim does not, at the same time, render the claim indefinite if each limitation recited in the claim is definite. *In re Wakefield*, 164 USPQ636 (CCPA 1970).

Reconsideration and withdrawal of the Section 112, 2nd paragraph rejection is respectfully requested.

Response to Section 102 Rejections over Srivastava (WO 95/24923) and Srivastava (US 5,961,979)

Claims 8-11 and 14-16 have been rejected as allegedly anticipated by Srivastava WO 95/24923 and by Srivastava US 5,961,979.

Applicant respectfully submits that the claims are not anticipated by the two Srivastava documents, for the following reasons.

Examiner submits that both WO95/24923 and US 5,961,979 of Srivastava disclose vaccine compositions comprising an immunogenic determinant which is comprised of a stress protein-peptide complex, isolated from a eukaryotic cell infected with a pre-selected intracellular pathogen, wherein the levels of the stress protein in the cell are increased upon the application of a range of stresses, including heat shock. Examiner submits that applicant's claims under examination are product-by-process claims and that "*if the product in [a] product-by-process claim is the same as or obvious from a product of the prior art, [then] the claim is unpatentable even though the prior [art] product was made by a different process*" and further that "*there does not appear to be structural difference between the product claimed and the product thought by the prior art*".

Applicant however maintains that there is an appreciable structural difference and distinction between the stress protein-peptide complexes of the instant invention and the stress protein-peptide complexes isolated from the cells used in WO 95/24923 and US 5961979 of Srivastava. Applicant respectfully points out that the claims of the instant invention, as amended, recite that the stress protein-peptide complexes of the instant invention comprise stress protein-peptide complexes which are not purified to homogeneity. That is, the stress protein complexes comprise a plurality of different stress protein species (for example a mixture containing a Hsp70-peptide complex, a Hsp60-peptide complex and a Hsp90-peptide complex). Accordingly, the stress-proteins comprised in the composition of the instant claims are heterogeneous.

This feature is taught by the instant specification at page 10, lines 20-26, which states: "*It will be appreciated that specific immunogenic SP/antigenic peptide fragment complexes can be isolated from the mixture of complexes produced from the stressing of the cellular material to produce a vaccine that is pathogen specific. However, this will usually not be required and the mixture of complexes can be used to induce broad spectrum immunisation.*" The same feature is

also stated at page 13, lines 21-24: "*It will be appreciated that the SP complexes isolated in this way are not purified to homogeneity, but are nevertheless suitable for use as a vaccine component*".

In contrast, both WO95/24923 and US 5,961,979 of Srivastava describe stress protein-peptide complexes purified to *homogeneity* via affinity chromatography. That is, the compositions of Srivastava consist of stress protein-peptide complexes comprised of a *single stress protein species only.* For example, see Srivastava (WO 95/24923) at the section entitled "*(a) Purification of Hsp70-peptide complexes*" at page 27, line 11 to page 28, line 22 of the WO95/24923, and in particular page 28, line 20, which states that "*the Hsp70-peptide complex can be purified to apparent homogeneity using this method*".

The particular antigenic peptide complexed to Hsp70 as disclosed in Srivastava will vary between different Hsp70 protein complexes, as the purification is Hsp70 specific. However, there will only be one stress-protein species present in the immunogenic composition of Srivastava, namely Hsp70.

A similar process is used in WO 95/24923 to purify the alternative stress protein species to homogeneity, since the same purification process is applied to all species. For further examples, see Hsp90-peptide complexes (page 30, lines 4-5) and gp96-peptide complexes (page 33, lines 4-5) also purified to "*apparent homogeneity*" by the method of WO 95/24923.

Similarly, US 5,961,979 of Srivastava employs purification techniques for the isolation of stress protein-peptide complexes from infected cells which are practically identical to those in WO 95/24923. In this methodology, the stress-protein peptide complexes are also purified to homogeneity. For example, column 13, second last paragraph entitled "*Purification of Hsp70-peptide complexes*", and in particular column 14, lines 30-31, "*The Hsp70-peptide complex can be purified to apparent homogeneity using this method*". A similar process is used in US 5,961,979 of Srivastava to purify the alternative stress protein species recited therein to homogeneity, since the same purification process is applied to all species. For further examples,

see Hsp90-peptide complexes (column 15, lines 9-10) and gp96-peptide complexes (column 15, lines 65-66) also purified to "*apparent homogeneity*" using the methods described in US 5,961,979.

In addition, applicant respectfully submits that the structural difference between the composition of the instant claims and those of Srivastava described above are further directly evident in the different types of immune responses elicited following administration of the compositions of Srivastava to a subject. In particular, the compositions comprising homogenous stress protein-peptide complexes of Srivastava disclosed in WO95/24923 and US 5,961,979 are only capable of eliciting cell-mediated cytotoxic T lymphocyte (CTL) responses. In contrast, it is a surprising discovery of the instant invention that the compositions comprising heterogeneous stress protein-peptide complexes are capable of eliciting *both* cell-mediated CTL responses *as well as* a humoral antibody response in the subject.

This capability is disclosed in the instant application, as filed. For example page 14, lines 8 to 17, makes it clear that the assessment of the immunogenicity of the stress protein-peptide complexes of the instant invention will involve examination of both T-cell and antibody responses. In contrast both WO95/24923 and US 5,961,979 of Srivastava are silent in relation to the elicitation of humoral immune responses in the subject. It is clear from a reading of the documents of Srivastava that the compositions used therein are for the elicitation of cell mediated CTL responses only. Indeed, both the documents of Srivastava are littered with statements to that effect.

For example page 9, lines 4-9 of WO95/24923 states that "*It has now been discovered that a subunit vaccine containing a stress protein peptide complex when isolated from cells infected with a pre-selected pathogen intracellular pathogen and then administered to a mammal can effectively stimulate cellular immune responses against cells infected with the same pathogen. Specifically, the immune response is mediated through the cytotoxic T cell cascade*". An identical statement is found at column 4, lines 55-68 of US 5,961,979.

Similarly, at page 9, lines 21-22 of WO95/24923, and at column 5, lines 5-7 of the US 5961979 patent the authors state that the preferred embodiment of both inventions comprises "*a vaccine that can be administered to a mammal for inducing in the mammal a cytotoxic T cell response*", and that "*The vaccines manufactured in accordance with the principles described herein contain an immunogenic stress protein-peptide complex that is capable of stimulating in the recipient a cytotoxic T cell response*" at page 9, lines 25-29 and column 5, lines 11-14 of the WO95/24923 and US 5961979 patents, respectively.

Moreover, all remaining embodiments of the inventions described throughout both Srivastava documents stipulate that the immune response generated is a cytotoxic T cell response. Other examples from WO95/24923 can be found at page 13, lines 1, 9, 12 and 14; at page 14, lines 5-9 which stipulate that "*the vaccine stimulates the cytotoxic T cell response via the major histocompatability complex (MHC) class I cascade*", which results in CTL responses only; at page 14, lines 12, 16 and 19; and at page 15, lines 12-15.

Examples from US 5,961,979 can be found at column 6, lines 54 and 65; at column 7, lines 2 and 5; at column 7, lines 30-35 which stipulate that "*the vaccine stimulates the cytotoxic T cell response via the major histocompatability complex (MHC) class I cascade*", which results in CTL responses only; at column 7, lines 38, 43-44, and 45-52; and at column 8, lines 6-12.

Thus, all contemplated embodiments of the inventions taught by WO95/24923 and US 5,961,979 of Srivastava are silent on the generation of humoral antibody responses and are concerned with the induction of cell mediated CTL immune responses only.

In contrast, Example 3 (at page 15, lines 20 to 23) of the instant application as filed discloses the generation of both CTL and humoral antibody responses in mice vaccinated with a composition containing complexes of the instant invention. Further, examples of good antibody responses elicited by the composition of the instant invention are provided in Example 4 (at page 17, lines 1-4) and Example 5 (at page 18, lines 10-12). In contrast all figures and examples disclosed in WO 95/24923 and US 5,961,979 of Srivastava are concerned only with CTL

responses. Hence, none of these examples demonstrate the generation of humoral antibody responses by the compositions of Srivastava.

In conclusion, there is therefore an inherent structural difference in the composition of the instant claims when compared to the compositions of WO95/24923 and US 5,961,979 of Srivastava for the reasons noted above. Applicant respectfully submits that the compositions of the instant invention are structurally distinct from the compositions of the prior art.

The technical problem solved by WO95/24923 and US 5961979 of Srivastava is the provision of an improved vaccine composition, comprising stress protein-peptide complexes, wherein a single stress protein species is purified to homogeneity, which is capable of inducing CTL responses, which are particularly favourable in the eradication and protection from intracellular pathogens. In contrast, the present application is based upon the surprising discovery by the applicant that the use of a composition comprising stress protein-peptide complexes, wherein a plurality of stress protein-peptide complexes comprised of multiple different species of stress protein are present, is capable of inducing a mixture of CTL and humoral antibody responses.

Without wishing to be bound by theory, applicant respectfully submits that it is the heterogeneous nature of the stress protein-peptide complexes present in the instant composition that results in this favourable mixed cell-mediated CTL and humoral antibody response. It is well known in the art that stress proteins have utility in directing exogenously provided antigens, which are normally processed via the major histocompatability complex (MHC) class II cascade, into the major histocompatability complex (MHC) class I cascade, which is normally reserved for endogenously provided or cytosolic antigens (see the document of Castellino *et al.* (1999), *J. Exp. Med.*, Jun 5, Vol. 191, No. 11, 1957-1964, a copy of which is hereby submitted in Appendix A). MHC II processing results in the generation of humoral immune responses and MHC I processing results in the generation of CTL responses. As such, stress proteins are capable of over-riding the normal antigen processing pathways and re-routing antigens in a process known as "cross presentation". Several mammalian stress protein-peptide complexes, when provided

exogenously, have been shown to re-route these exogenous antigens away from the MHC II cascade, and into the MHC I cascade, resulting in the generation of CTL responses to the peptide antigens in place of the normally expected antibody responses (see for example the documents of Suto and Srivastava (1995), *Science*, Sep 15, Vol. 269, 1585-1589; Singh-Jasuja *et al.* (1999), *J. Exp. Med.*, Jun 5, Vol. 191, No. 11, 1965-1974, copies of which are submitted in Appendix A).

More recently, however, it has been shown that certain stress proteins exist in distinct isoforms, some of which are capable of preferentially directing exogenous antigens into the MHC II cascade, resulting in the generation of efficient antibody responses to exogenous antigens (see the abstract of Houlihan *et al.* (2009), *J. Immunol.*, Jun 15;182(12):7451-8, a copy of which is submitted in Appendix A). It is clearly demonstrated in the instant application as filed, in contrast to the compositions of Srivastava disclosed in WO95/24923 and US 5,961,979, that the composition of the instant invention is capable of generating both cell mediated CTL and humoral antibody responses. The applicant submits that it is the mixture of heterogeneously purified stress protein-peptide complexes contained in the composition of the instant invention that results in a wider array of stress proteins being presented to the cells of the immune system, said complexes comprising a larger number of distinct isoforms of different heat shock proteins, which are capable of efficient direction of the peptide antigen which is complexed with the heat shock protein into *both* the MHC I *and* MHC II cascades when compared to the compositions of Srivastava. Accordingly, protective cell mediated CTL and humoral antibody responses are elicited in the subject following administration of the composition of the instant invention. In contrast, the homogenously purified stress protein-peptide complexes of Srivastava are capable of directing peptide antigen into the MHC I cascade *only*. Thus only cell-mediated CTL responses are generated by these compositions.

Furthermore, applicant submits that a further structural differences between the stress protein-peptide complexes of the instant claims and those disclosed in WO95/24923 and US 5961979 of Srivastava are evident from the methods of manufacture employed in the production of the different compositions. Specifically, applicant respectfully submits that the method of production of stress protein-peptide complexes taught by Srivastava in both WO95/24923 and

US 5,961,979 results in the purification of complexes wherein the stress protein component of the complexes is derived from the host cell only. In contrast, the compositions of the instant invention comprise stress protein-peptide complexes wherein the stress proteins comprise a mixture of a plurality of different host cell-derived and bacterial-derived stress proteins. Further, applicant submits that that these bacterial derived stress-proteins have particular utility at directing peptide antigens to the MHC II cascade.

During the processing of extracellular antigens and/or pathogens, the extracellular antigens and/or pathogens are phagocytosed by antigen presenting cells (APC) into endosomes, which then fuse with lysosomes to produce phagolysosomes, wherein the antigen/pathogen is digested into peptides. The phagolysosome then fuses with a MHC II containing vesicle that buds off the endoplasmic reticulum, and the peptides are loaded onto MHC II molecules, which are then presented by MHC II on the cell surface. This is known as the "exocytic" antigen processing pathway. The APC then matures, migrates to the local lymph nodes (LLN) and presents the antigen (via MHC II) to naive T cells located within the lymph node. The naive T cells then clonally expand into Th1 and Th2 cells expressing a T cell receptor specific for the peptide which is presented by the MHC II on the APC. Th2 cells then direct the induction of humoral antibody immune responses from B cells.

In contrast, intracellularly-derived or cytosolic pathogens/antigens are processed separately in the cytosol and are loaded onto MHC I molecules and presented on the cell surface. This is known as the endocytic antigen processing pathway, which results in the generation of cell-mediated CTL responses only.

The method of production of the stress protein-peptide complexes taught in WO 95/24923 and US 5,961,979 of Srivastava employs the use of a dounce homogenizer to rupture cells (for example at page 27, line 19 in the WO 95/24923 patent, or at column 13, lines 60-63 in US 5961979), or alternatively a mild lysis buffer which importantly does *not* comprise detergent (this lysis buffer is described at page 27, line 13-15 in WO95/24923, and at column 13, lines 54-57 in US 5,961,979). In both of the documents of Srivastava, intracellular bacteria contained

within endosomes would not be lysed by these methods. The lysis of the bacterial cell wall is well known in the art to require the use of detergent, multiple freeze-thaw cycles, lytic enzymes such as lysozyme, strong physical forces such as sonication or a French press, or high osmotic/ionic pressures. In many cases, it is necessary to employ several of these strategies either simultaneously or in succession in order to affect efficient cell lysis of bacterial cells.

In the case of the Srivastava documents, none of these methods are employed. Moreover, following lysis by the dounce homogenizer in both WO 95/24923 and US 5,961,979, the lysate is centrifuged, first at low speed to remove unbroken cells and debris, and immediately again at ultra-high speed at 100,000g (for example see page 27, lines 22-24 in WO 95/24923, or column 13, lines 64-67 in US 5,961,979). It is the supernatant from this latter centrifugation step that is used for purification of the stress protein-peptide complexes in the methods of Srivastava.

It is well known in the art that such high centrifugation speeds are used to pellet viruses, bacteria and intracellular organelles, and therefore allow their separation from the supernatant which contains the stress protein-peptide complexes. Thus, the supernatant from this step that is loaded onto an affinity purification column, as taught at page 27, line 29 of WO 95/24923, or at column 14, lines 1-2 of US 5,961,979, would contain *only* host cell-derived stress proteins. The non-lysed intracellular bacteria and endosomes would be efficiently pelleted by the ultra-high speed centrifugation step at 100,000g. Hence, neither bacterial stress proteins nor bacterial stress protein-peptide complexes would be present in the system following purification on the affinity column.

In contrast, the method of production of the stress protein-peptide complexes disclosed in the instant application as filed employs the use of a dounce homogenizer *and a buffer comprising the detergent Tween* (see Example 1, page 13, lines 1-6 of the application as filed). In this example, upon disruption of the host cells the detergent would efficiently solubilize the endosomal membranes and those of the intracellular bacteria contained therein, resulting in the release of bacterial-derived stress protein-peptide complexes *in addition to* host cell-derived stress protein-peptide complexes into the lysate. Similarly, Example 2 of the application as filed

discloses the use of a dounce homogenizer; cycles of freeze-thaw or detergent lysis (see Example 2, page 15, lines 1-3 of the application as filed). Hence, as outlined above, these methods would also result in the liberation of bacterial-derived as well as host-cell derived stress protein-peptide complexes into the lysate, as both the detergent and freeze-thaw techniques are capable of lysing bacterial cells. Further, Example 5 details the use of disruption of infected host cells with 1 % Tween detergent solution (see Example 5, page 17, and lines 22-24 of the application as filed).

Thus, in all embodiments of the instant invention, the pooling of pathogen-derived and host-derived stress proteins is disclosed, and a further inherent structural difference between the compositions of the instant invention and those of Srivastava therefore exists.

Moreover, applicant submits that it would be obvious to the skilled person on reading WO95/24923 and US 5,961,979 that the compositions of Srivastava were intended to solely comprise host cell-derived stress proteins. Indeed Srivastava is silent on the use of bacterial stress proteins for the elicitation of humoral immune responses. In contrast, ample disclosure of the use of both host-cell derived and bacterial-derived stress protein-peptide complexes can be found in the instant application as filed, for example at page 9 lines 3-5 "*any suitable pathogen-infected cell or cell line can be used in the present invention, to provide a source of SP complexes*" and at lines 28-30 "*preferably the vaccine contains a plurality of SP/antigenic peptide fragment complexes derived from the stressed pathogen*".

As detailed above, applicant submits that the mixture of bacterial-derived and host-cell derived stress protein-peptide complexes comprised in the composition of the instant claims, in contrast to the compositions of Srivastava, provides a wider array of stress proteins, which have capability in generating a mixture of both CTL and humoral immune responses in a subject. Without wishing to be bound by theory, applicant submits that these bacterial derived stress-proteins have particular utility at directing peptide antigens to the MHC II cascade (see the document of Tobian *et al.* (2004), *J. Immunol.* 173: 5130-5137, a copy of which is submitted in Appendix A). In addition, there is recent evidence within the field demonstrating that APC can distinguish between self and non-self derived stress-protein-peptide complexes, which further

implies the species-specific nature of the immune responses generated by stress protein-peptide complexes (see the last two lines of the abstract of Binder, RJ (2009), *J. Immunol.* 182(11): 6844-50, a copy of which is submitted in Appendix A).

In summary, applicant submits that the induced stress protein complexes of the present invention are *structurally distinct* from the stress protein-peptide complexes disclosed by Srivastava, because the compositions of the instant invention comprise a mixture of different stress-protein complexes, such mixture comprising a wider array of different isoforms of stress-proteins, in addition to pathogen-derived stress proteins, which are capable of inducing both cell-mediated CTL and humoral antibody responses. Simply put, such a product is not anticipated by either of WO 95/24923 and US 5,961,979.

Reconsideration,and withdrawal of the Section 102 rejection of claims 8-11 and 14-16 over Srivastava WO95/24923 and US 5,961,979 is respectfully requested.

Response to Section 102 Rejections over Wallen *et al.* (US 5,747,332) and Laminet *et al* (EMBO (1990). 9(7): 2315-2319)

Claims 8-11 and 14-16 have been rejected as allegedly anticipated by Wallen *et al.*, and by Laminet *et al.*

Examiner submits that both Wallen and Laminet disclose the isolation of stress protein-peptide complexes, including those comprised of the bacterial stress proteins GroEL/ES. The stress protein-peptide complexes can be produced by heat treatment of cells, and can be used as the immunogenic determinant in vaccine compositions. Examiner submits that applicant's claims under examination are product-by-process claims and that "*if the product in [a] product-by-process claim is the same as or obvious from a product of the prior art, [then] the claim is unpatentable even though the prior [art] product was made by a different process*" and that "*there does not appear to be structural difference between the product claimed and the product thought by the prior art*".

Applicant however maintains that there is an appreciable structural difference and distinction between the stress protein-peptide complexes of the instant invention and the stress protein-peptide complexes taught in both the Wallen and Laminet prior art documents. Applicant respectfully points out that both Wallen and Laminet, similar to WO 95/24923 and US 5,961,979 of Srivastava, teach the isolation of stress protein-peptide complexes from cells derived from one cell type only. In contrast, as outlined above, the method of production employed in the instant invention results in the isolation of a mixture of both host cell-derived and pathogen-derived stress protein-peptide complexes.

In this regard, Wallen states at column 2, lines 54-55 "*typically the solution from which the heat shock protein complexes are purified is a cell lysate from a tumor in which the HSP's are already present*". Indeed, Example 1 of Wallen details the purification of stress protein-peptide complexes from B16-F1 mouse melanoma cells (see Example 1, column 4, lines 12-13). Thus, the stress protein-peptide complexes of Wallen are host cell-derived *only*. There is no discussion in Wallen of the infection of such cells, or indeed the presence of intra-cellular pathogens in those cells, as is required by the claims of the instant invention. The only mention of pathogen derived stress protein-peptide complexes made in Wallen is located at column 3, lines 54-55. In this particular case, mention of GroEL/GroES is made for the sole purpose of illustrating that these proteins belong to the Hsp60 family. Simply put, there is no disclosure or teaching in Wallen whatsoever of a product comprising a mixture of bacterial and mammalian derived stress-protein-peptide complexes.

Similarly, the prior art of Laminet is also solely concerned with the production of stress-protein-peptide complexes isolated from one cell type *only*, in contrast to the present invention which concerns the production of stress-protein-peptide complexes isolated from both host cells and pathogen cells. Laminet only describes the stress protein-peptide complex of the single bacterial stress protein GroEL/ES complexed to the peptide β-lactamase. There is no disclosure or mention whatsoever in Laminet of a mixture of several different stress proteins, of both bacterial and eukaryotic cell origin, or their use as effective immunogenic determinants in

vaccine compositions, in contrast to the composition of the instant invention. Further, there is no mention in Laminet that the antigenic peptide fragment is derived from an intracellular pathogen.

In conclusion, the applicant respectfully submits that there is an inherent structural difference between the product of the instant claims when compared to those of the prior art, as applicant's product involves as immunogenic determinant a plurality of different isoforms and species of stress proteins complexed to peptide antigen, comprised of both pathogen-derived and host cell derived stress proteins, such a product being capable of eliciting both cell-mediated and humoral immune responses in a subject. Such a product is simply not taught by any of the prior art documents cited.

Response to Obviousness-Type Double Patenting Rejection

Claims 8-11 and 14-16 are provisionally rejected on the ground of non-statutory obviousness-type double patenting as being unpatentable over claims 10-14 and 17 of copending Application No. 10/049,704, on the basis that the co-pending claims are not patentably distinct.

Applicant respectfully submits that the claims as amended require that the antigenic peptide fragment of the stress protein/antigenic peptide fragment complex is derived from the intracellular pathogen which is infecting the host cell. As such, the claims of the instant application are limited to peptide fragments derived from an intracellular pathogen. Co-pending Application No. 10/049,704 on the other hand requires the antigenic peptide fragment to be derived from an extracellular pathogen. Hence, it is respectfully submitted that the claims are patentably distinct from the allegedly conflicting claims of Application No. 10/049,704. Reconsideration and withdrawal of the obviousness-type double patenting rejection of claims 8-11 and 14-16 is respectfully requested.

Status of Other Applications of Applicant Directed to Related Subject Matter

Application No. 10/049,704. The '704 application was subject to an office action dated December 12, 2008. A response to that action was filed on June 12, 2009.

Application No. 10/363,454 (US-2005-0232946): The '704 application was subject to an office action dated June 2, 2009. Applicant has not yet responded to the office action.

Conclusion

The claims of the application are believed in condition for allowance. An early action toward that end is earnest solicited.

Respectfully submitted

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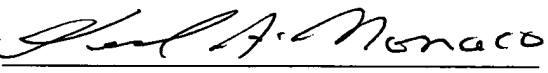
BY 

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APPENDIX A

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Receptor-mediated Uptake of Antigen/Heat Shock Protein Complexes Results in Major Histocompatibility Complex Class I Antigen Presentation via Two Distinct Processing Pathways

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Abstract

Heat shock proteins (HSPs) derived from tumors or virally infected cells can stimulate antigen-specific $CD8^+$ T cell responses in vitro and in vivo. Although this antigenicity is known to arise from HSP-associated peptides presented to the immune system by major histocompatibility complex (MHC) class I molecules, the cell biology underlying this presentation process remains poorly understood. Here we show that HSP 70 binds to the surface of antigen presenting cells by a mechanism with the characteristics of a saturable receptor system. After this membrane interaction, processing and MHC class I presentation of the HSP-associated antigen can occur via either a cytosolic (transporter associated with antigen processing [TAP] and proteasome–dependent) or an endosomal (TAP and proteasome–independent) route, with the preferred pathway determined by the sequence context of the optimal antigenic peptide within the HSP-associated material. These findings not only characterize two highly efficient, specific pathways leading to the conversion of HSP-associated antigens into ligands for $CD8^+$ T cells, they also imply the existence of a mechanism for receptor-facilitated transmembrane transport of HSP or HSP-associated ligands from the plasma membrane or lumen of endosomes into the cytosol.

Key words: immunology • vaccines • macrophages • T cells • peptides

Introduction

Activation of the T cell limb of the adaptive immune system involves the recognition of small ligands bound to MHC or MHC-like proteins on plasma membranes. This antigen presentation paradigm is best understood for peptides associated with MHC class I or class II molecules, with a large body of work having defined two distinct pathways by which protein antigens are converted to shorter fragments occupying the polymorphic binding domains of these MHC glycoproteins (1). For MHC class I molecules, peptides are mainly created by the action of proteasomal enzymes on proteins present in the cytosol. These peptides are then transported by the transporter associated with antigen processing (TAP)[1] dimer through the membrane of the endoplasmic reticulum (ER), after which they are bound to nascent MHC class I heavy chain–β2-microglobulin complexes within the ER lumen (2). The occupied MHC molecules then pass through the secretory pathway to the cell surface where they can interact with the receptors of $CD8^+$ T cells. MHC class II molecules instead interact primarily with large polypeptides within various endosomal compartments

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[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; HSP, heat shock protein; TAP, transporter associated with antigen processing.

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(3–5), after which exopeptidases remove the unprotected segments of the antigen outside of the MHC molecule binding groove (6, 7). The resulting MHC class II–peptide complexes are then exported to the plasma membrane for recognition by $CD4^+$ T cells.

Heat shock proteins (HSPs) are highly conserved peptide-binding molecules that control the folding of proteins and prevent their aggregation (8). The same peptide-binding capacity appears to allow HSPs to acquire proteinaceous antigenic material within cells, and when administered exogenously, to induce priming of $CD8^+$ T lymphocytes in vivo (9–13). This property suggests that there is efficient transfer of antigen from HSPs to MHC class I molecules under these conditions. Such transfer is surprising both because peptide loading of MHC class I molecules typically involves a cytosol to ER route, and because while we understand the role of several well-defined receptor systems in facilitating MHC class II–peptide presentation (14–18), little is known about any specific pathways for capture and delivery of exogenous antigens to (intracellular) MHC class I molecules. In circumstances other than those known to involve HSP (19–22), MHC class I presentation of peptides derived from exogenous protein sources has been reported and ascribed to either endosomal degradation of particulate antigens, followed by "regurgitation" of the derived peptides for loading of surface class I molecules (23), or to delivery of antigens from endosomes and/or phagosomes into the cytosol by an unknown route (24–27). The relative contribution of either of these proposed pathways to presentation of HSP-associated antigen has not been determined, nor is much known about the cell biology of such exogenous pathways for MHC class I–peptide acquisition.

Several groups have recently reported that various HSP molecules show cell surface binding and/or cellular uptake that in some (28, 29) but not other (30) cases has the characteristics expected of a saturable receptor system. However, these studies did not link these binding events to functional antigen presentation, or address by what route the antigen in the bound HSP is converted into peptides associated with MHC class I molecules. Given other studies showing active signaling by HSP for induction of cytokine secretion by various cell types (31), it was possible that the binding reported in these earlier investigations was related to this functional stimulation and not necessarily to antigen presentation. Here, we analyze HSP delivery of antigenic material to MHC class I molecules using both functional and morphologic methods. Our data reveal that saturable uptake of the complexes, apparently through interaction with one or more surface receptors, is important for delivering HSP-associated antigen to the cell for processing via either an endosomal or a cytosolic route. Which processing pathway is preferred appears to depend on the sequence context of the antigenic peptide bound to the HSP. The evidence presented here for proteasome-dependent processing of HSP-derived material also implies the existence of an uncharacterized mechanism for transport of HSP complexes, or their cargo, across plasma or endosomal membranes into the cytosol.

Materials and Methods

Cells. Macrophages were collected by peritoneal lavage 6 d after injection of 1 ml of thioglycollate into C57BL/6 or (C3H × C57BL/6)F1 mice (The Jackson Laboratory). B3Z is a $CD8^+$ T cell hybridoma specific for OVA 257–264 (SIINFEKL) bound to H-$2K^b$ MHC class I molecules (32). 3A9 is a $CD4^+$ T cell hybridoma specific for the hen egg lysozyme determinant corresponding to residues 46–61 bound to the MHC class II molecule I-A^k (33).

HSP. Recombinant HSP 70 protein was expressed from the pMS236 plasmid constructed by cloning the 1.96-kb NcoI-XbaI fragment of the genomic mouse clone hsp70.1 (34) into the pTrc99 expression vector (Amersham Pharmacia Biotech). Expression in DH5α cells was induced by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), harvested cells were disrupted, and clarified supernatants were loaded onto a DEAE Sephacel column (Amersham Pharmacia Biotech). Elution of HSP 70 was achieved by applying a 25–500 mM NaCl gradient. Fractions containing HSP 70 were loaded onto an ATP-agarose column (Sigma-Aldrich), and elution was achieved by applying a 0–1 mM ATP gradient. Fractions containing HSP 70 were precipitated with 80% ammonium sulfate, redissolved in low salt buffer containing magnesium acetate, dialyzed against the same buffer, and aliquots were stored at −80°C. Bovine HSP 70 was purchased from Sigma-Aldrich.

HSP/Antigen Complexes. The loading of mouse HSP 70 was performed as described previously (11) with minor variations. The HSP and the hybrid peptide (synthesized by Bio-Synthesis, Inc.) were incubated together in PBS containing 1 mM KCl, 2 mM $MgCl_2$, and 100 μM ATP for 45 min at room temperature. 1 mM ADP was added, and the incubation was extended for an additional 30 min. The free hybrid peptide was removed by extensive washing using a Centricon 30K (Amicon), until the residual free hybrid peptide was calculated to be in the picomolar range. The efficiency of the loading was determined with iodine-labeled hybrid peptide to be ~20% in most preparations.

Bioassays. 10^5 thioglycollate-induced macrophages were cultured overnight in each well of a flat-bottomed 96-well plate in the absence or presence of the indicated antigens and 5×10^4 B3Z or 3A9 cells in a final volume of 200 μl. As an indication of the T cell activation, IL-2 accumulation in the supernatant at 16 h was measured by ELISA (BD PharMingen) (26). For the competition experiments, macrophages were preincubated with the indicated amount of unloaded bovine HSP 70 (Sigma-Aldrich) for 30 min at 37°C in a final volume of 100 μl. Without washing, loaded HSP 70 or antigenic peptide was then added along with 5×10^4 B3Z cells, and incubation continued for an additional 16 h before assay of IL-2 accumulation. Error bars show SEM; where no bars are visible, the errors were too small to plot.

Lactacystin Treatment. (C57BL/6 × C3H)F1 macrophages were cultured for 8 h with the indicated antigens in the presence or absence of 25 μM lactacystin (Calbiochem). The APCs were then fixed with 1% paraformaldehyde for 10 min at room temperature, treated for 10 min with 0.1 M glycine, washed, and cultured overnight with either B3Z or 3A9 T cell hybridoma cells. IL-2 accumulation in the supernatant at 16 h was measured as an indication of T cell activation.

Immunofluorescence Staining. Bovine HSP 70 (Sigma-Aldrich), BSA (Sigma-Aldrich), and 25-D1.16 (an mAb specific for the SIINFEL/K^b complex [see reference 41]) were biotinylated using *N*-hydroxy succinimide–biotin (Pierce Chemical Co.) according to the manufacturer's directions. FITC-OVA (Molecular Probes) was used at a concentration of 200 μg/ml. Rabbit anti-

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calnexin (StressGen Biotechnologies) and FITC-conjugated mouse anti-rabbit antibodies (Jackson ImmunoResearch Laboratories) were used at 5 μg/ml. Steptavidin-Texas red (Southern Biotechnology Associates) was used at 2 μg/ml. Biotinylated 25-D1.16 was used at 10 μg/ml. Details of intracellular staining analyzed by immunofluorescence confocal microscopy are given in the legend to Fig. 3. Details of the surface staining and internalization assay, analyzed by immunofluorescence confocal microscopy, are given in the legend to Fig. 4. In brief, 5×10^5 thioglycollate-induced macrophages were grown overnight in 24-well plates on poly-L-lysine (Sigma-Aldrich) coated coverslips. The next day, the coverslips were washed to remove the nonadherent cells and incubated with the indicated antigens in 250 μl of complete medium. Cells were fixed with 1% paraformaldehyde (see Fig. 3) or with 3% paraformaldehyde (see Fig. 4) followed by quenching with 0.1 M glycine. Unless otherwise indicated, cells were permeabilized with 0.1% Brij. In Fig. 4 A, cells were stained with Streptavidin-Texas red without permeabilization. Coverslips were mounted with Fluorosave (Calbiochem). Digital images were acquired using a Leica LSCM, composed in Adobe Photoshop®, and printed after formatting in ClarisDraw.

Saturation and Competition Binding Assays. For saturation binding analysis, 5×10^5 thioglycollate-induced macrophages were incubated with increasing concentrations of either biotinylated HSP 70 or biotinylated BSA for 45 min on ice, then washed. For the binding competition assay, macrophages were incubated first with the indicated amount of unlabeled HSP 70 or BSA for 45 min on ice, and followed without washing by incubation with biotinylated HSP 70 for an additional 45 min. Cells were washed two times and incubated with Streptavidin-PE (Molecular Probes) and FITC-CD11b (BD PharMingen), then washed again. Data were collected using a FACScan™ flow cytometer (Becton Dickinson) after addition of propidium iodide and analyzed using CELLQuest™ software (Becton Dickinson). Gates were set for $CD11b^+$, propidium iodide–negative cells, and either flow histograms of HSP staining of $CD11b^+$ cells staining were generated (competition experiment), or mean fluorescence values of HSP staining for this cell subpopulation were calculated (saturation analysis).

Results

Differential Roles of the Proteasome and TAP in MHC Class I Presentation of SIINFEKL Present in Two Distinct HSP Complexes. To gain a better understanding of HSP antigenicity, with its implications for both the basic biology of antigen presentation and the development of HSP-based vaccines, we first addressed whether the proposed "regurgitation" route or the cytosol to ER route, or both, contributes to the presentation of HSP-associated antigen. This was accomplished by examining the effects of inhibiting proteasome activity or eliminating TAP function on T cell stimulation by HSP/antigen complexes. Mouse HSP 70 was loaded in vitro with synthetic antigens containing the amino acid sequence SIINFEKL (residues 257–264 of OVA) that is presented by H-$2K^b$. The binding motif of HSP 70 consists of a hydrophobic core of four to five amino acids and two flanking regions enriched in basic residues (8, 35). Because SIINFEKL does not contain this motif, we synthesized hybrid peptides containing a sequence derived from BiP that binds avidly to HSP 70 and that is connected to SIINFEKL via a GSG linker at the antigenic peptide's NH_2 terminus (HWDFAWPWGSGSIINFEKL, referred to as BiP-OVA) or the converse (SIINFEKLGSGHWDFAWPW, referred to as OVA-BiP). The two different orientations of SIINFEKL and BiP motif were tested because the sequence context of a MHC class I–binding determinant within a protein has been shown to affect its efficiency of presentation (36, 37). With both of these hybrid peptides, the loading efficiency of HSP 70 was typically 20%, versus only 1% occupancy using antigenic peptides not optimized for HSP binding (11; and data not shown). These HSP/hybrid peptide complexes are highly effective at priming CTL responses in vivo (38).

In the presence of macrophages as APCs, HSP 70 loaded with either BiP-OVA (HSP/BiP-OVA) or OVA-BiP (HSP/OVA-BiP) stimulated IL-2 secretion from the SIINFEKL/K^b-specific T cell hybridoma B3Z, whereas neither hybrid peptide added alone to macrophages was effective (Fig. 1). This excludes the possibility that activation achieved using HSP/hybrid peptide complexes is mediated by residual free peptide and suggests that an active cellular process is necessary for generation of the SIINFEKL/K^b ligand. HSP 70 itself, or HSP 70 mixed with hybrid peptides under conditions that do not support stable complex formation, was not immunogenic. Taking into account the 20% loading, the HSP/antigen complexes were as efficient on a molar basis as the potent optimal-length free SIINFEKL peptide, despite their requirement for additional processing.

The role of proteasomal enzymes in the presentation of SIINFEKL from the HSP 70/antigen complexes was examined by incubating peritoneal macrophages with either of the HSP/hybrid peptide complexes in the presence of the potent proteasomal inhibitor lactacystin (39). Only stimulation by HSP/OVA-BiP, but not by HSP/BiP-OVA, was substantially inhibited by lactacystin used at 25 μM, a concentration that did not influence presentation of free SIINFEKL peptide or of an endosomally processed determinant of hen egg lysozyme that binds to MHC class II molecules (Fig. 2 A). Such selective inhibition argues against either a general toxic effect or a nonspecific inhibi-

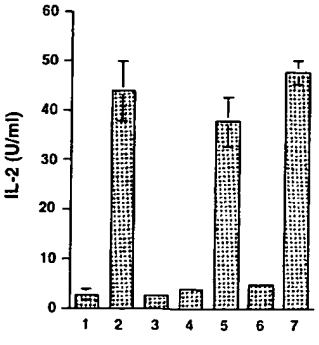

Figure 1. Antigenic peptides derived from material bound to HSP 70 in vitro can be presented by MHC class I molecules of normal macrophages. C57BL/6 macrophages were cultured overnight in (1) the absence of any antigen, (2) the presence of 1 nM SIINFEKL peptide, (3) 2 μg of unloaded HSP 70, (4) 20 nM OVA-BiP, (5) 2 μg HSP70/OVA-BiP, (6) 20 nM BiP-OVA, or (7) 2 μg HSP 70/BiP-OVA complexes and 5×10^4 B3Z. IL-2 accumulation in the supernatant at 16 h was measured as an indication of T cell activation. Comparable results were obtained in >10 similar experiments.

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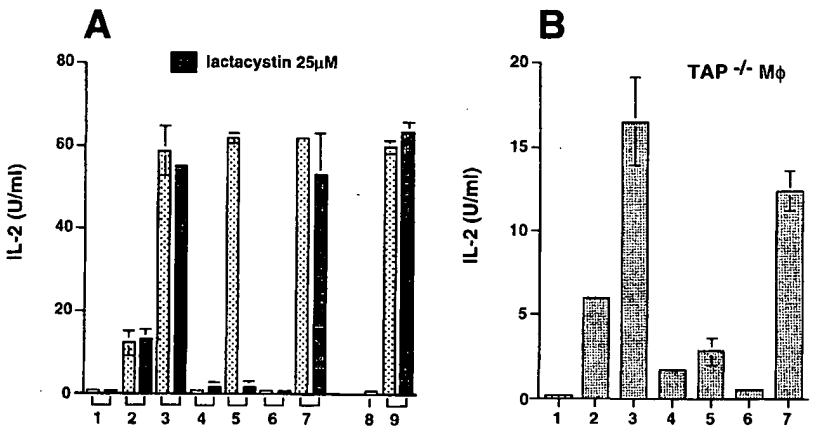

Figure 2. Presentation of SIINFEKL from HSP/OVA-BiP but not HSP/BiP-OVA is dependent on proteasomal proteases and TAP. (A) The effect of 25 μM lactacystin on the presentation of the indicated antigens to either the B3Z or the 3A9 T cell hybridoma cells was assayed. In bars 1–7, the presentation to B3Z hybridoma cells was analyzed in (1) the absence of any antigen, (2) the presence of 1 nM SIINFEKL peptide, (3) 10 nM SIINFEKL peptide, (4) 20 nM OVA-BiP, (5) 2 μg HSP70/OVA-BiP, (6) 20 nM BiP-OVA, or (7) 2 μg HSP 70/BiP-OVA complexes. In four independent experiments, the presentation of HSP 70/BiP-OVA was not affected by lactacystin, whereas the inhibition of the presentation of HSP70/OVA-BiP ranged between 70 and 90%. The effect of lactacystin on the presentation of HEL-46-61 to 3A9 hybridoma cells was tested (8) in the absence of any antigen, or (9) in the presence of 1 mg/ml hen egg lysosyme. (B) 10^5 TAP-$1^{-/-}$ macrophages (C57BL/6 × 129) were grown at 26°C for 24 h and transferred to 37°C when incubation with the indicated antigens and the T cells was started. The presentation to B3Z hybridoma cells was analyzed in (1) the absence of any antigen, (2) the presence of 1 nM SIINFEKL peptide, (3) 10 nM SIINFEKL peptide, (4) 20 nM OVA-BiP, (5) 2 μg HSP70/OVA-BiP, (6) 20 nM BiP-OVA, or (7) 2 μg HSP 70/BiP-OVA complexes. Similar results were obtained in three independent experiments.

tion of cellular proteases, and suggests that HSP can use two distinct intracellular processing pathways to deliver antigenic peptides to MHC class I molecules, only one of which involves proteasome activity. To further define these pathways, we tested the role of TAP. TAP-deficient macrophages express lower levels of MHC class I molecules at the cell surface than wild-type cells, but this limitation can be overcome by growing the cells at reduced temperature in β2-microglobulin (40). TAP$^{-/-}$ macrophages were therefore grown at 26°C for 24 h and transferred to 37°C when incubation with the antigens and the T cells was started. In agreement with the data obtained using lactacystin, TAP-deficient macrophages showed greatly impaired presentation of SIINFEKL from HSP/OVA-BiP but not from HSP/BiP-OVA (Fig. 2 B).

Direct Visualization of the Sites of SIINFEKL-K^b Binding in Cells Exposed to HSP/Antigen Complexes. These functional experiments suggest that HSP/OVA-BiP is preferentially processed via the classical class I pathway and imply that the HSP/ligand complex, or the ligand alone, is translocated into the cytosol after which proteasomal cleavage and TAP transport contribute to MHC class I loading of released peptide in the ER. In contrast, HSP/BiP-OVA appears to be predominantly processed by a distinct pathway. To visualize directly the site(s) of SIINFEKL-MHC class I association after exposure of cells to HSP/antigen complexes, macrophages were incubated with each of the HSP complexes, and the cells were stained with 25-D1.16, an mAb to SIINFEKL/K^b complexes (41). Compared with cells exposed to HSP only (not shown), we could detect SIINFEKL/K^b complexes with 25-D1.16 when macrophages were incubated with either of the HSP/antigen preparations. In agreement with the implications of the antigen presentation studies, the intracellular distribution of these MHC class I/peptide complexes varied depending on the antigen employed. With HSP/BiP-OVA, whose presentation is insensitive to proteasomal inhibition and is TAP independent, a vesicular staining predominates (Fig. 3 A). 25-D1.16 staining (Fig. 3 C) partially colocalizes with OVA internalized by fluid phase endocytosis (Fig. 3, D–E), indicating that with this HSP/antigen combination, SIINFEKL-loaded MHC class I molecules are found preferentially in endosomes. In contrast, when macrophages are pulsed with HSP/OVA-BiP, the staining of vesicular structures is infrequent (Fig. 3 B), and the 25-D1.16 staining (Fig. 3 F) largely colocalizes with calnexin used to identify the ER (Fig. 3, G and H). Together with the above data on proteasome and TAP dependence of HSP/OVA-BiP presentation, these staining data support the existence of an HSP-mediated pathway for the translocation of polypeptides into the cytosol, in agreement with a recent study involving a lymphoma cell line (30).

These functional and morphologic results thus reveal that two distinct routes of processing can lead to MHC class I presentation of HSP-associated antigenic material, and also suggest that which pathway is preferentially used depends on the relationship of the class I binding sequence to the surrounding peptide structure. Having a free COOH-terminal anchor residue (BiP-OVA) seems to be associated with effective processing by an endosomal route. In contrast, having this key binding residue within an extended polypeptide chain (OVA-BiP) appears to require proteasomal action for efficiently placing this amino acid at the COOH terminus of the processed material (42, 43). Additional peptide sequences will need to be tested to determine if this is truly a general rule, which would have important implications for the design of HSP-based vaccines.

Possible Role of Receptor-mediated Uptake in HSP-dependent Antigen Presentation. The efficiency of presentation using HSP/hybrid peptide complexes suggests the existence of a specific mechanism that can maximize the available pool of antigenic substrate within the cell. Several recent studies have provided evidence for receptor-mediated binding of various HSPs to cell surfaces (28) and/or receptor-mediated uptake into endosomes (28, 29). To look for evidence of a receptor uptake system under the conditions employed

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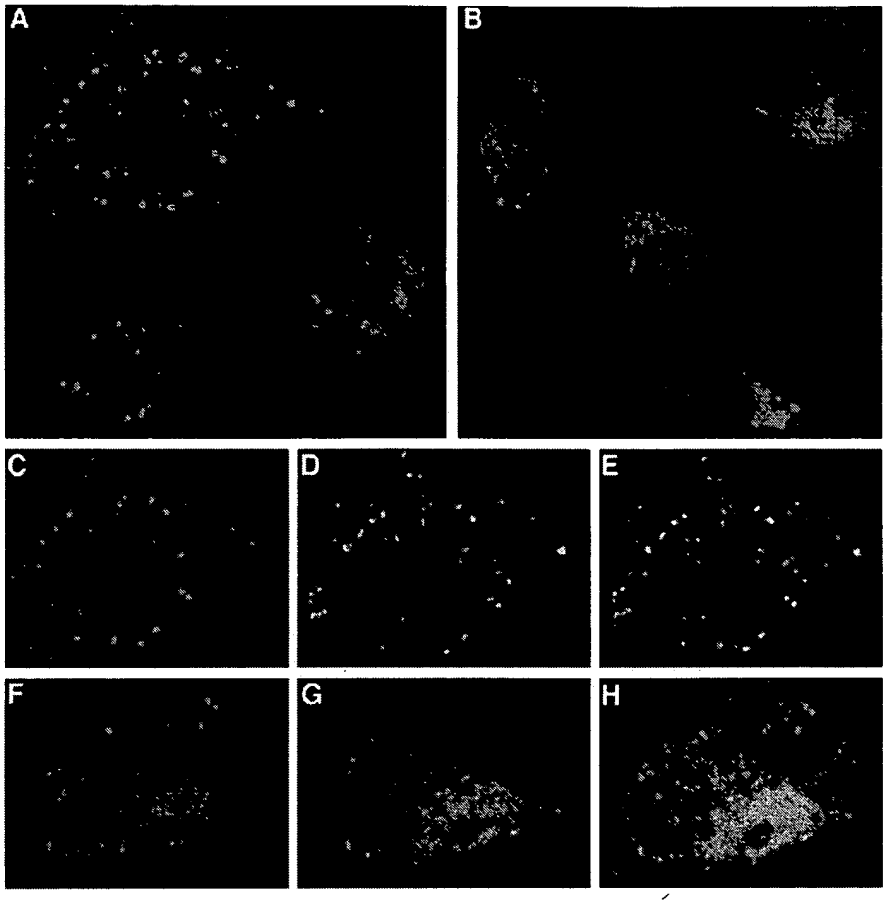

Figure 3. Direct visualization of two distinct intracellular sites of SIINFEKL association with MHC class I molecules after antigen delivery via HSP 70. C57BL/6 macrophages were incubated for 6 h at 37°C with either 100 μg/ml of HSP/BiP-OVA (A, C–E) or HSP/OVA-BiP (B, F–H). During the last 30 min of incubation, FITC-OVA was added to some of the samples. A, B, C, and F show the red channel fluorescence of cells stained with biotinylated 25-D1.16 followed by Streptavidin–Texas red. C shows the red channel (SIINFEKL/K^b complexes) for one cell, demonstrating the predominant vesicular location of these complexes; D shows the green channel (FITC-OVA) for the same high-power field; and E shows the colocalization of the two signals (yellow), indicating the endosomal nature of at least a fraction of the SIINFEKL/K^b-containing structures. In F–H, cells were double stained with biotinylated 25-D1.16 followed by Streptavidin–Texas red as well as with rabbit anticalnexin followed by FITC-conjugated anti-rabbit Ig. F shows the red channel (SIINFEKL/K^b complexes), revealing primarily reticular cytoplasmic staining along with a few stained vesicles. G shows the green channel (calnexin) for the same cell. Colocalization of the two signals (yellow) is seen in H, consistent with an ER location for the bulk of the SIINFEKL/K^b complexes.

here, we examined the interaction of biotinylated HSP with peritoneal macrophages. Incubation with biotinylated HSP 70 at 4°C resulted in binding to the cell surface (Fig. 4 A). The bound HSP was internalized into endocytic vesicles when the cells were warmed to 37°C (Fig. 4 B), where it partially colocalized with OVA provided as a fluid phase marker (Fig. 4, C and D). This binding and rapid endocytic uptake were specific to HSP 70, as biotinylated BSA gave no surface staining at 4°C and little concentration in vesicles after a longer time at 37°C under these conditions (not shown). These data are in agreement with and extend recent results showing that glucose-regulated protein (Grp) 96 and heat shock cognate protein (HSC) 70 can bind to the plasma membrane and internalize into endosomal-like structures (28, 29).

The surface binding observed microscopically could also be detected using flow cytometry. Only 25–30% of isolated peritoneal macrophages, a population composed largely of the CD11b-brightest cells (data not shown), exhibited substantial cell surface binding of biotinylated HSP 70 (Fig. 4 E). Interestingly, this matches the proportion of HSP-pulsed macrophages that can be lysed by CTLs specific for HSP-associated antigen (12). Thus, the level of surface receptor expression correlates with and may determine effective presentation of HSP-associated ligand. Consistent with the existence of a saturable, specific receptor system, surface binding of biotinylated HSP to the $CD11b^{bright}$ cells could be partially but substantially inhibited by a 10-fold excess of unlabeled HSP, but not by BSA (Fig. 4 E), and exposure to increasing concentrations of HSP 70 resulted in staining approaching a plateau at 200 μg/ml among the $CD11b^{bright}$ cells (Fig. 4 F). A 10-fold excess of unloaded HSP (100 μg/ml) also substantially inhibited B3Z responses to either of the HSP/hybrid peptide complexes (Fig. 4 G), in accord with the idea that the receptor-mediated binding observed by staining contributes to antigen presentation by both processing pathways detailed above.

Discussion

The evidence provided here for both a cytosolic and endocytic route for MHC class I presentation of HSP-associated antigen provides new mechanistic insight into a well-recognized functional property of HSP. The two pathways we have directly visualized correspond to those previously proposed to underlie presentation of material associated with artificial particulate antigens or with bacteria (23–27). In our own previous study of latex bead–associated protein, the efficiency of MHC class I–dependent antigen presentation was low, which we considered inconsistent with the operation of a highly evolved specific pathway (26). In contrast, HSP-mediated antigen presentation is quite effi-

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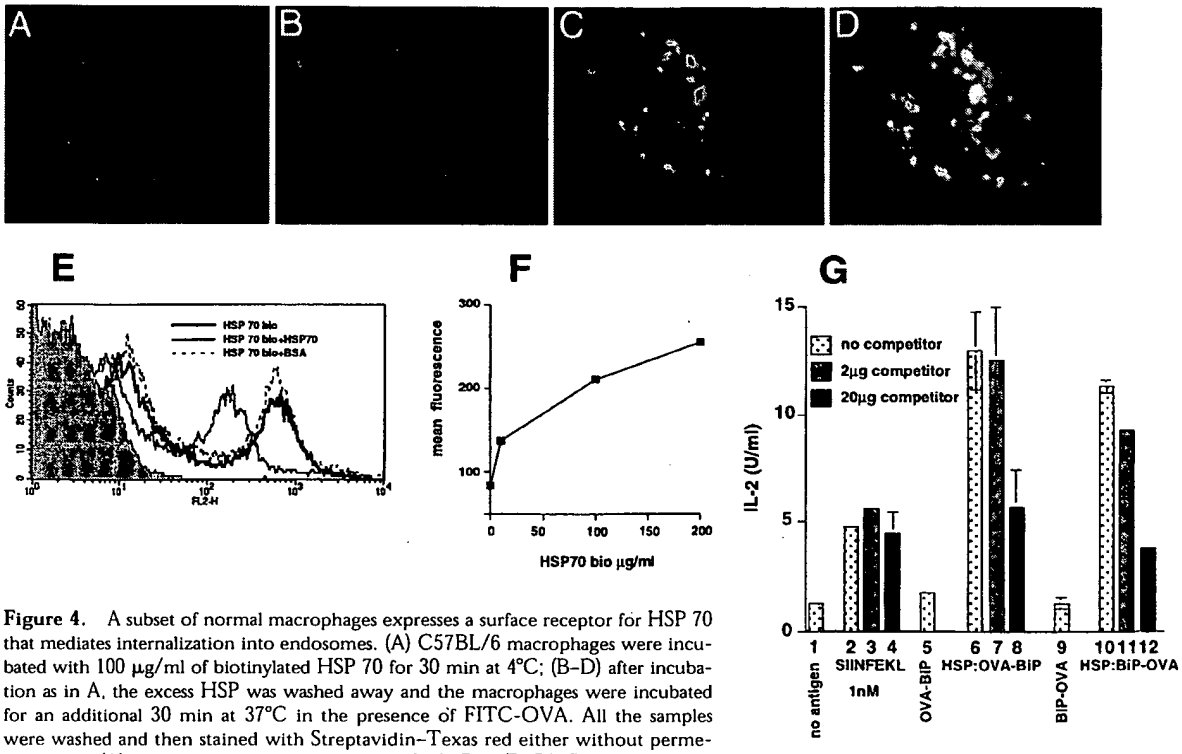

Figure 4. A subset of normal macrophages expresses a surface receptor for HSP 70 that mediates internalization into endosomes. (A) C57BL/6 macrophages were incubated with 100 μg/ml of biotinylated HSP 70 for 30 min at 4°C; (B–D) after incubation as in A, the excess HSP was washed away and the macrophages were incubated for an additional 30 min at 37°C in the presence of FITC-OVA. All the samples were washed and then stained with Streptavidin–Texas red either without permeabilization (A), or after permeabilization with 0.1% Brij (B–D). B shows the red channel (HSP 70), and C shows the green channel (FITC-OVA). D shows the partial colocalization of the two signals (yellow). (E) 3×10^5 macrophages were incubated at 4°C with 50 μg/ml of biotinylated BSA (gray histogram), with 50 μg/ml of biotinylated HSP 70 (thick solid line), or with 500 μg/ml of unlabeled HSP 70 for 30 min, followed without washing by addition of 50 μg/ml of biotinylated HSP 70 and incubation for an additional 30 min (thin solid line), or with 500 μg/ml of unlabeled BSA for 30 min, followed without washing by addition of 50 μg/ml of biotinylated HSP 70 and incubation for an additional 30 min (dotted line). Cells were stained with FITC-anti-CD11b and streptavidin-PE. Only propidium iodide–negative $CD11b^+$ cells are shown. (F) 5×10^5 macrophages were incubated at 4°C with increasing concentrations of biotinylated HSP 70 or biotinylated BSA. Cells were washed and stained with FITC–anti-CD11b and Streptavidin-PE. Only propidium iodide–negative $CD11b^+$ cells are included in the analysis. The mean fluorescence of the $CD11b^{bright}$ subpopulation of macrophages that shows high binding to HSP 70 is plotted. (G) Macrophages were cultured overnight in the presence or absence of the indicated antigens and 5×10^4 B3Z cells. Where indicated "competitor," macrophages were preincubated for 30 min with 2 or 20 μg of unloaded HSP 70 in 100 μl of medium, followed without washing by addition of 2 μg of antigen-loaded HSP or SIINFEKL and B3Z cells. In four independent experiments, the inhibition of the presentation of either HSP70/OVA-BiP or HSP 70/BiP-OVA by 20 μg of competitor averaged 50%.

cient and our congruent microscopic and functional evidence support the existence of a specific receptor uptake system for HSP/antigen complexes, extending both morphological data and extensive quantitative uptake data from other recent studies (28, 29) by showing a functional relationship between such receptor-dependent surface binding and HSP-dependent MHC class I antigen presentation.

Receptor-mediated uptake improves MHC class II antigen presentation by up to four orders of magnitude (14–18). The presumably similar enhancing effects of such uptake on the HSP-dependent presentation pathway, together with the expression of these putative receptors on subsets of professional APCs involved in initiating immune responses (including splenic dendritic cells [Castellino, F., unpublished results]), strongly imply that class I, and possibly also class II, MHC molecule presentation of HSP-associated antigens is a physiologically relevant pathway. The heterogeneity seen for HSP 70 binding to peritoneal mononuclear cells suggests that expression of the HSP binding structures is regulated during cellular differentiation, whereas the continued small increment in staining observed at very high HSP concentrations (>200 μg/ml; our unpublished observations) raises the possibility that more than one affinity class of receptor may exist. The staining at very high HSP concentrations could reflect weak cross-binding among the different receptors proposed by Arnold-Schild et al. to mediate Grp 96 versus HSP 70 binding (28).

Antigen cross-presentation plays a key role in responses to tumors and possibly to virally infected nonhematopoietic cells, as well as in the establishment of peripheral tolerance (44). The mechanism of antigen acquisition and the cells involved in in vivo presentation in these circumstances are poorly understood. Uptake of apoptotic cells by phagocytic dendritic cells has been suggested as a key event (22), but recent data favor the activation of dendritic cells by material from necrotic cells (45), followed by efficient antigen presentation of released material by MHC class I molecules (22, 46). The loss of HSP/protein complexes from cells dying a necrotic death could be readily imagined to lead to

antigen delivery via the processing pathways described here, in agreement with the proposal of Melcher et al. (47). Some antigen-associated HSP might also be released from viable infected or transformed cells due to saturation of the KDEL-retrieval pathway (48) after the HSP upregulation that results from stress due to microbial invasion or the poor oxygenation of growing tumors. In these cases, the complexes could contribute to an "early warning" system that promotes T cell activation by professional APCs that have efficiently bound them through surface receptors and converted them into peptide/MHC molecule ligands suitable for T cell recognition.

Finally, from a cell biology point of view, we are left with trying to understand how HSP/antigen complexes or the antigenic cargo of HSP molecules move across endosomal or plasma membranes into the cytosol. The only described pathway across such membranes for normal cellular proteins is in the opposite direction and involves lysosomes (49). Whether the same pathway can operate in reverse or whether another transport pathway remains to be discovered poses an intriguing question.

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ated cardiac damage in normal mice is not the result of viral-mediated cytolysis, but rather is from the actions of recruited inflammatory cells. The data are also consistent with, but do not prove, a requirement of MIP-1α in an autoimmune-mediated model of CVB3-induced myocarditis. In this regard, it is noteworthy that antibodies to MIP-1α reduce the severity of experimental autoimmune encephalomyelitis (8), and that the murine MIP-1α gene maps to a region of chromosome 11 that includes the Idd4 insulin-dependent diabetes locus (13).

We infected −/− mice with influenza virus to examine the inflammatory response to a pathogen of different tissue tropism. This model was chosen because pulmonary alveolar macrophages are a rich source of MIP-1α (14), suggesting that it may be involved in pulmonary inflammation. Influenza-infected mice were killed at day 6 or 7 p.i., when pathology is maximum (15). The lungs of most of the +/+ animals were inflamed and edematous; those of the −/− mice were less severely affected. Histologic sections of lung were graded for inflammation between 0+ and 4+ (16), on the basis of the extent of mononuclear cell infiltration and tissue damage (Fig. 3). The +/+ mice had significantly more inflammation than −/− mice ($P = 0.012$ by the Wilcoxon rank order test). These data demonstrate that MIP-1α contributes to influenza virus–induced pneumonitis.

Influenza virus is generally cleared from the lungs of immunocompetent mice by 6 to 10 days p.i. by a T cell–dependent mechanism (17). To determine whether MIP-1α affects influenza virus clearance, we measured viral titers at various times after infection and calculated the geometric mean for each genotype (Fig. 3D). Viral titers were higher in −/− animals compared with +/+ controls at day 3 p.i. ($P = 0.14$ by the two-tailed t test) and significantly so at days 6 and 7 p.i. ($P = 0.02$). Part of this difference may be accounted for by the increased weight of the +/+ lungs, but this weight difference was at most twofold, whereas the difference in viral titers was 100- to 1000-fold. By day 21 p.i., no virus was detected in either group of mice. This delay in T cell–dependent viral clearance in the −/− mice suggests that MIP-1α may be required for efficient recruitment of immunocompetent T cells to sites of viral infection, which is consistent with the ability of the chemokine to induce chemotaxis of T cells in vitro (2, 3).

Our experiments provide genetic evidence for the requirement of a β chemokine in inflammation and demonstrate that other chemokines do not functionally substitute for MIP-1α. The relative importance of MIP-1α in an inflammatory response may depend on the pathogen or tissue involved, possibly because of the expression of other molecules with compensatory activity. Nevertheless, the role of MIP-1α in diseases as different as myocarditis and pneumonitis suggests that this chemokine may also mediate inflammation in response to a variety of other stimuli.

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A Mechanism for the Specific Immunogenicity of Heat Shock Protein–Chaperoned Peptides

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Endogenously synthesized antigenic determinants are generally presented on major histocompatibility complex (MHC) class I molecules, whereas exogenous determinants are presented by MHC class II molecules. Here, it is shown that exogenous antigens chaperoned by a heat shock protein can be channeled into the endogenous pathway, presented by MHC class I molecules, and recognized by $CD8^+$ T lymphocytes. This pathway is functional only in a subset of macrophages among the cell types tested. These observations provide a basis for the tumor-specific and virus-specific immunogenicity of cognate heat shock protein preparations and offer a mechanism for the classical phenomenon of cross-priming.

Heat shock proteins (HSPs) isolated from cancer cells or virus-infected cells elicit protective immunity or cytotoxic T lymphocytes (CTLs) to the cognate tumor or viral antigen (1–3). In contrast, HSPs isolated from normal tissues do not elicit such immunity (2, 3). Because the HSP genes do not show tumor-associated DNA polymorphism, it has been suggested that HSPs derived from cancers or virus-infected cells are not immunogenic per se but rather chaperone tumor- or virus-specific antigenic peptides generated during antigen processing, and that it is the peptides and not the HSPs that are immunogenic (4). This suggestion was upheld by the sequencing of a number of HSP-associated peptides (5) and by the observation that HSPs stripped of associated peptides lose their immunogenicity (3).

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One of the unresolved questions in this area has been the mechanism whereby HSP-peptide complexes elicit specific immunity. Immunogenicity of HSP preparations is exquisitely dependent on the presence of functional phagocytic cells in the host; the depletion of such cells rendered the host incapable of being immunized by HSP preparations (6). This observation led to the suggestion that HSPs are taken up by the macrophage and are re-presented by the MHC class I molecules of the macrophage, which finally stimulate the appropriate T cells (7). Thus, a mechanism of indirect presentation of HSP-chaperoned peptides was invoked. The observations reported here support this mechanism.

We have investigated whether HSP-chaperoned peptides could be re-presented by phagocytic cells in a vesicular stomatitis virus (VSV) model. The HSP gp96 was isolated to apparent homogeneity (8) from EL4 cells transfected with the gene encoding the

.nucleocapsid protein of VSV (N1 cells) (9). As a negative control, gp96 was also isolated from untransfected EL4 cells. Because soluble and particulate moieties are taken up by macrophages in essentially different ways, the gp96 preparations were centrifuged at 100,000g for 90 min to ensure that only soluble gp96 was used. The gp96 preparations were used to pulse macrophages in vitro that were obtained from the peritoneal exudate cells of C57BL/6 mice. We measured the ability of the pulsed macrophages to stimulate VSV-specific, K^b-restricted CTLs as a function of their ability to stimulate CTLs to release tumor necrosis factor (TNF) and their ability to act as targets of such CTLs. Macrophages pulsed with N1-derived gp96 could stimulate the release of TNF by VSV-specific CTLs, whereas those pulsed with EL4-derived gp96 could not (Fig. 1A). In cytotoxicity assays, macrophages pulsed with N1-derived gp96 could be lysed by VSV-specific CTLs, whereas those pulsed with EL4-derived gp96 were not (Fig. 1B). The quantity of N1-derived gp96 necessary to elicit a half-maximal response was found to be the same (1.5 to 3 μg/ml) for the two assays in any given experiment. Lysis of gp96-pulsed macrophages was inhibited by antibodies to K^b or to CD8, but not by antibodies to D^b or to CD4 (Fig. 1C).

The proportion of macrophages that were lysed never exceeded 30%, even at high effector-to-target (E/T) ratios (Fig. 2B). This persistently limited lysis suggests that only a subpopulation of macrophages can internalize and present HSP-chaperoned peptides in the context of MHC class I molecules. This idea is reminiscent of the observations of Rock and colleagues, who suggested that a small subpopulation of splenic macrophages can present exogenous antigens through the endogenous pathway (*10*). The possibility that only a specific macrophage population can present HSP-chaperoned peptides is supported by the observation that the macrophage line RAW309.Cr.1, which is functional in phagocytosis and pinocytosis and in antigen presentation to MHC class II–restricted T helper cells, could not present gp96-chaperoned peptides to VSV-specific CTLs (*11*). B cells and fibroblasts were also tested for their ability to present gp96-chaperoned peptides through the MHC class I molecules and were found to be unable to do so (*11*).

The above data indicate that N1-derived gp96 molecules are chaperoning the K^b epitope of VSV (or a precursor thereof) and that this epitope is being inducted into the endogenous presentation pathway of the macrophages. We tested this premise directly with the use of gp96 preparations reconstituted in vitro with the octamer K^b-binding epitope of VSV (VSV8). Macrophages pulsed with VSV8-gp96 complex, but not those pulsed with peptide A–gp96 complex, were recognized by VSV-specific CTLs (Fig. 2, A and B). Interestingly, a longer 20-mer peptide containing the K^b epitope, which cannot sensitize macrophages for CTL recognition by itself, did so quite effectively—and to an extent comparable to VSV8—when complexed with gp96 (Fig. 2C). To determine whether complexes of peptides with any protein might be able to sensitize macrophages for specific recognition, we tested peptides complexed with albumin or histone as in Fig. 2, A and B; no sensitizing effect was detected (*11*). A quantitative comparison of the ability of free and gp96-chaperoned VSV8 to stimulate CTLs showed that VSV8-gp96 complexes were 200 to 400 times more efficient at pulsing macrophages than was VSV8 alone; a free VSV8 concentration of ~50 nM was necessary to elicit the same level of CTL stimulation by pulsed macrophages as was elicited by 200 pM VSV8-gp96 complex (Fig. 2D).

We next examined the sensitivity of the macrophages' re-presentation of gp96-chaperoned peptides to a number of inhibitors (Table 1). Depletion of the intracellular adenosine triphosphate pools of the macro-

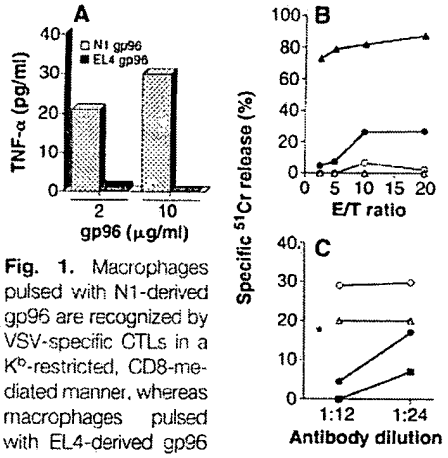

Fig. 1. Macrophages pulsed with N1-derived gp96 are recognized by VSV-specific CTLs in a K^b-restricted, CD8-mediated manner, whereas macrophages pulsed with EL4-derived gp96 are not. (**A**) Pristane-induced macrophages (1×10^4) from C57BL/6 mice and VSV-specific CTLs (5×10^4) were cocultured in the presence of gp96 (2 or 10 μg/ml) derived from N1 or EL4 cells in 96-well U-bottom plates at 37°C. After 24 hours, supernatants were collected and TNF-α production was measured by bioassay in a cytotoxicity assay with WEHI164 cells (*17*). (**B**) The ability of gp96-pulsed macrophages to act as targets in CTL assays was tested. Macrophages (5×10^5 per milliliter) were pulsed with gp96 (10 μg/ml) derived from N1 cells (●) or EL4 cells (○), with VSV nucleocapsid K^b epitope peptide (10 μM) (▲) as a positive control, or with a medium control (△) for 2 hours at 37°C, followed by labeling with ^{51}Cr for 1.5 hours. These cells were used as targets in a 4-hour ^{51}Cr release assay with VSV-specific CTLs. (**C**) CD4 monoclonal antibody (mAb GK1.5) ascites (○) (obtained from E. Nakayama, Okayama University School of Medicine, Okayama, Japan), CD8 mAb (YTS169.4) (■), H-2K^b mAb (Y3) (●), H-2D^b mAb (B22.249) (△), or RPMI 1640 control (*) were added to the CTL assay at the same time as effector cells and ^{51}Cr-labeled macrophages pulsed with N1 gp96 (E/T ratio = 10). See (*18*) for additional specificity analysis.

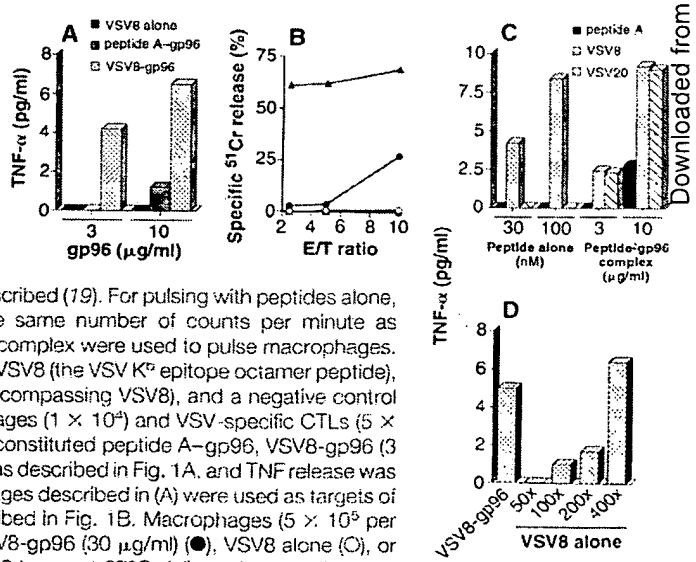

Fig. 2. Macrophages pulsed with in vitro reconstituted complexes of gp96 with the K^b epitope of VSV are recognized by VSV-specific CTLs. Macrophages were pulsed with free peptides or in vitro reconstituted complexes of gp96 with experimental or control peptides, as described (*19*). For pulsing with peptides alone, free peptides containing the same number of counts per minute as present in the gp96-peptide complex were used to pulse macrophages. The peptides used (*20*) were VSV8 (the VSV K^b epitope octamer peptide), VSV20 (a 20-mer peptide encompassing VSV8), and a negative control VSV peptide A. (**A**) Macrophages (1×10^4) and VSV-specific CTLs (5×10^4) were cocultured with reconstituted peptide A–gp96, VSV8-gp96 (3 or 10 μg/ml), or VSV8 alone, as described in Fig. 1A, and TNF release was monitored. (**B**) The macrophages described in (A) were used as targets of VSV-specific CTLs, as described in Fig. 1B. Macrophages (5×10^5 per 100 μl) were pulsed with VSV8-gp96 (30 μg/ml) (●), VSV8 alone (○), or liver gp96 (30 μg/ml) (■) for 2 hours at 37°C, followed by labeling with ^{51}Cr for 1.5 hours. They were then used as targets in a 4-hour ^{51}Cr release assay with VSV-specific CTLs. As a positive control, N1 cells were used as targets (▲). (**C**) Free and gp96-complexed VSV8 and VSV20 and gp96-complexed peptide A (negative control) were used to pulse macrophages at 3 and 10 μg/ml, and their ability to stimulate VSV-specific CTLs was monitored. (**D**) The ability of VSV8-gp96 complexes to pulse macrophages so as to enable them to stimulate CTLs was compared quantitatively to the corresponding ability of free VSV8 alone. Macrophages were pulsed with VSV8-gp96 complex (3 μg of gp96 per milliliter, or 31 nM) or the indicated relative quantities of free VSV8 (1× free VSV8 concentration refers to the concentration of VSV8 present in the VSV8-gp96 complex at 3 μg/ml and is equivalent to ~200 pM). TNF production by sensitized macrophages was measured as described in Fig. 1.

phages by pretreatment with sodium azide and 2-deoxyglucose was inhibitory, whereas chloroquine, which inhibits processing in an acidic microenvironment, had no effect. In contrast, pretreatment of macrophages with brefeldin A (BFA) abrogated re-presentation. Apparently, gp96-peptide complexes are internalized by macrophages (that is, processed through nonacidic compartments) and the gp96-peptide complexes or the peptides alone are routed through the endoplasmic reticulum (ER) by a mechanism dependent on (*10*) or independent (*12*) of transport-associated proteins.

Several lines of evidence indicate that gp96-chaperoned peptides are processed internally and are re-presented by the MHC class I molecules of the macrophages, and that the effects reported here are not a consequence of surface phenomena: (i) Sensitization of surface MHC class I molecules is limited by the size of peptide, whereas the gp96-chaperoned peptides show no such preference. (ii) The gp96-chaperoned peptides are several hundred times as effective as free peptides in sensitizing macrophages for CTL recognition. (iii) Although free peptides sensitize all cell types for CTL recognition, sensitization by gp96-chaperoned peptides is limited to a subset of macrophages. (iv) The processing of gp96-peptide complexes in the macrophages is sensitive to BFA. Collectively, these observations begin to shed light on the long-standing puzzle of how immunization of mice with HSP-peptide complexes elicits tumor-specific and virus-specific CTL response (*1–3*).

Together with the observations that HSPs bind antigenic peptides and that the ER-resident HSP gp96 associates with MHC class I molecules (*13*), the lack of allelic variation in HSP genes (*14*) had previously led us to predict that HSP-associated peptides would serve as precursors of peptides associated with any given MHC class I and that HSPs will be able to cross-prime (*7*). This prediction has been tested by two independent approaches. In the first approach, gp96 isolated from VSV-infected cells of $H\text{-}2^b$ haplotype (EL4 cells) and $H\text{-}2^d$ haplotype (Meth A cells) was used to sensitize macrophages (of $H\text{-}2^b$ haplotype) for recognition by VSV-specific CTLs of $H\text{-}2^b$ haplotype. The gp96 preparations isolated from VSV-infected cells of either haplotype were equally efficient at sensitization (Fig. 3A). In the second approach, gp96 preparations from VSV-infected cells of the $H\text{-}2^b$ or $H\text{-}2^d$ haplotypes were used to immunize $H\text{-}2^b$ haplotype mice and the mice were tested for $H\text{-}2^b$–restricted CTL response. The gp96 preparations from VSV-infected cells of either haplotype elicited a specific CTL response (Fig. 3B). These experiments indicate that peptides chaperoned by gp96 are indeed unselected with respect to the MHC haplotype. Further, as predicted (*7*), these experiments demonstrate cross-priming by HSP preparations and suggest that HSPs can act as mediators of this classical phenomenon (*15*).

Table 1. The re-presentation of gp96-chaperoned peptides by macrophages is inhibited by BFA and by sodium azide plus 2-deoxyglucose, but not by chloroquine. Inhibitors were used as described (*16*).

Pretreatment	Pulsing with VSV8		Pulsing with N1 gp96	
	Cytotoxicity (%)	Inhibition (%)	Cytotoxicity (%)	Inhibition (%)
	Experiment 1			
None	90.0		26.0	
BFA	85.0	5.6	6.0	76.9
	Experiment 2			
None	94.0		26.2	
Azide + 2-deoxyglucose	74.5	20.7	0	100
Chloroquine	75.0	20.2	22.3	14.2

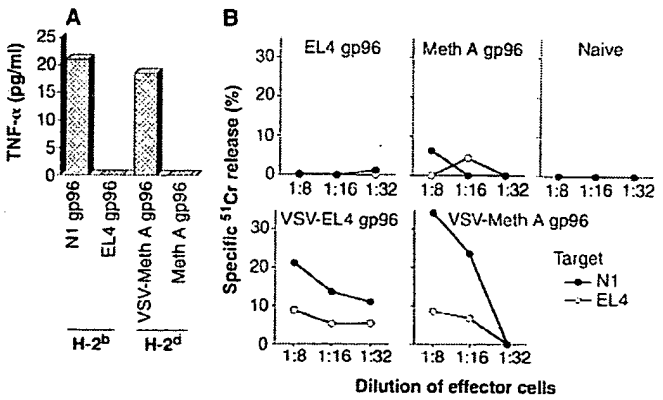

Fig. 3. (A) The gp96 preparations isolated from VSV-infected cells of the $H\text{-}2^b$ or $H\text{-}2^d$ haplotypes can be processed by macrophages of the $H\text{-}2^b$ haplotype to stimulate K^b-restricted VSV-specific CTLs. Macrophages ($H\text{-}2^b$) were pulsed with gp96 (3 μg/ml) derived from uninfected or VSV-infected Meth A ($H\text{-}2^d$) cells or from EL4 ($H\text{-}2^b$) or N1 ($H\text{-}2^b$) cells and were used to stimulate K^b-restricted CTLs as described in Fig. 1A. **(B)** Preparations of gp96 isolated from VSV-infected cells of the $H\text{-}2^b$ or $H\text{-}2^d$ haplotypes can prime mice of the $H\text{-}2^b$ haplotype for generation of VSV-specific CTLs. C57BL/6 mice ($H\text{-}2^b$) were vaccinated with gp96 derived from uninfected or VSV-infected Meth A ($H\text{-}2^d$) or EL4 ($H\text{-}2^b$) cells. Spleens of immunized mice were tested for cytotoxicity against N1 cells or EL4 cells (*21*).

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16. Macrophages (5×10^6 per milliliter) were preincubated with sodium azide (30 mM) plus 2-deoxyglucose (5 mM) for 1.5 hours, followed by pulsing with N1 gp96 (10 μg) or VSV8 (10 μM) for 2 hours at 37°C. The pulsed cells were labeled with ^{51}Cr for 1.5 hours and used as target cells in a 4-hour ^{51}Cr release assay with VSV-specific CTLs in the absence of inhibitors. For inhibition with chloroquine (100 μM), cells were similarly treated except that the CTL assay was carried out for only 2 hours; this duration was predetermined to be sufficient to detect significant CTL response. Also, the effect of chloroquine was not fully reversible in this duration, as determined by the use of an MHC class II–restricted line against an unrelated antigen. In experiments with BFA (1.5 μg/ml), the inhibitor was present throughout the experiment, although its concentration was reduced by half during the CTL assay. This concentration of BFA was predetermined to have no direct inhibitory effect on CTLs. E/T ratios were 12 for experiment 1 and 20 for experiment 2.
17. WEHI164 cells were seeded (2500 cells per well) in flat-bottom 96-well plates. Serially diluted supernatants of macrophage-CTL coculture were added; recombinant TNF-α was added to WEHI164 in separate wells as control. After 4 hours of culture at 37°C, 50 μl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 1 mg/ml) was added, with 4 hours of incubation, followed by 100 μl of propanol and 0.05 M HCl. Optical density (590 nm) was measured immediately. Sample concentrations were calculated by comparison with dilution points, which resulted in killing of 50% of WEHI164 cells.
18. We also tested the specificity of the ability of gp96-

pulsed macrophages by using the macrophages pulsed with either gp96 preparation to stimulate CTLs against an irrelevant tumor. None of the pulsed macrophages could stimulate the tumor-specific CTLs.

19. For in vitro reconstitution of gp96-peptide complexes, gp96 derived from normal liver (50 μg) and ^{125}I-labeled peptides (5 μg) were incubated at 50°C for 10 min followed by room temperature for 30 min. Free peptides were removed by extensive washing with Microcon 50 (Amicon), such that no free peptides were detected on SDS–polyacrylamide gel electrophoresis of the complexes (Z. Li, R. Suto, P. K. Srivastava, in preparation).
20. The sequence of VSV20 is Ser-Leu-Ser-Asp-Leu-Arg-Gly-Tyr-Val-Tyr-Gln-Gly-Leu-Lys-Ser-Gly-Asn-Val-Ser-Cys. The sequence of the negative control VSV peptide A is Lys-Arg-Gln-Ile-Tyr-Thr-Asp-Leu-Glu-Met-Asn-Arg-Leu-Gly-Lys.
21. C57BL/6 mice (*H-2*^{b} haplotype) were subcutaneously injected twice at a 7-day interval with gp96 (10 μg in phosphate-buffered saline) derived from uninfected or VSV-infected Meth A cells or EL4 cells. Seven days after the second vaccination, spleens were removed and spleen cells (8 × 10^{6} cells per well) were cocultured in mixed lymphocyte-tumor culture (MLTC) with irradiated N1 cells (1.4 × 10^{5} cells per well) in 24-well plates. On day 7, each well was harvested. Serially diluted culture cells were tested against N1 cells or EL4 cells for cytotoxicity in a ^{51}Cr release assay.
22. Supported by NIH grant CA44786. R.S. is a postdoctoral fellow of the Cancer Research Institute, New York.

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Human H-Y: A Male-Specific Histocompatibility Antigen Derived from the SMCY Protein

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H-Y is a transplantation antigen that can lead to rejection of male organ and bone marrow grafts by female recipients, even if the donor and recipient match at the major histocompatibility locus of humans, the HLA (human leukocyte antigen) locus. However, the origin and function of H-Y antigens has eluded researchers for 40 years. One human H-Y antigen presented by HLA-B7 was identified as an 11-residue peptide derived from SMCY, an evolutionarily conserved protein encoded on the Y chromosome. The protein from the homologous gene on the X chromosome, *SMCX*, differs by two amino acid residues in the same region. The identification of H-Y may aid in transplantation prognosis, prenatal diagnosis, and fertilization strategies.

Histocompatibility antigens that can induce transplant rejection include the class I and class II molecules of the major histocompatibility complex (MHC), as well as a large number of so-called minor histocompatibility (H) antigens. In mice, the use of inbred strains has shown that minor H antigens are encoded by almost 50 different allelically polymorphic loci scattered throughout the genome (*1*). Humans also have minor H antigens although their overall number and complexity remains uncertain. Both species have the male specific antigen H-Y (*2*, *3*). H-Y was initially identified through the observation that within an inbred mouse strain, most of the male-to-female skin grafts were rejected, whereas transplants in other sex combinations nearly always succeeded (*2*). In humans, sex mismatch is a significant risk factor associated with rejection or the development of graft-versus-host disease in bone marrow transplant recipients (*3*–*6*). The H-Y antigen is expressed in most different human tissues (*4*, *7*), and H-Y specific immune responses occur during the transplantation of other organs, blood transfusion, and pregnancy (*8*).

As with other minor H antigens, the recognition of H-Y by T lymphocytes is MHC-restricted (*3*, *9*), and some H-Y antigens are peptides derived from cellular proteins that are presented on the cell surface in association with MHC class I molecules (*10*). We have developed a technique for the identification of individual peptides that are bound to MHC molecules and recognized as antigens by T cells. By combining microcapillary liquid chromatography–electrospray ionization mass spectrometry with T cell epitope reconstitution assays (*11*–*13*) we now report the identification of a peptide antigen recognized by a human cytotoxic T lymphocyte (CTL) clone that is H-Y–specific and restricted by the class I MHC molecule HLA-B7.

To isolate endogenously processed H-Y peptides, HLA-B7 molecules were purified by affinity chromatography from the H-Y positive, B lymphoblastoid cell line JY (*14*). The associated peptides were extracted in acid and separated from high molecular weight material by ultrafiltration (*15*) and subsequently fractionated by reverse-phase high-pressure liquid chromatography (HPLC) (*11*). Samples of each fraction were incubated with HLA-B7^{+}, H-Y^{-} T2-B7 target cells to assay for reconstitution of the epitope recognized by

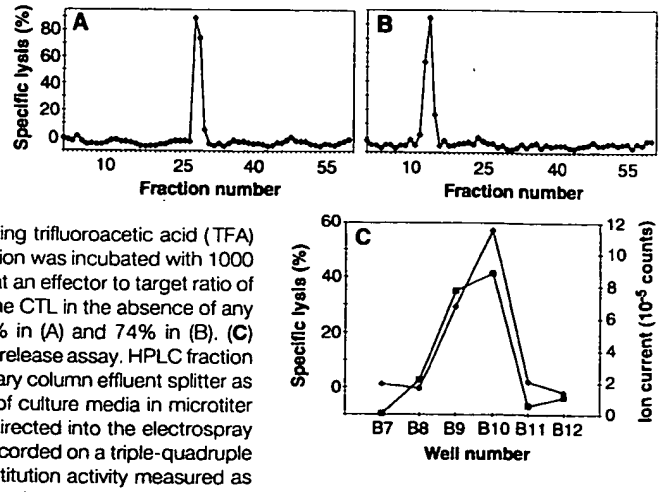

Fig. 1. Reconstitution of the H-Y epitope with HPLC-fractionated peptides extracted from HLA-B7 molecules. (**A**) HLA-B7-molecules were immunoaffinity purified form 2 × 10^{10} H-Y^{+} JY cells. Peptides were eluted from B7 molecules with 10% acetic acid, pH 2.1, filtered through a 5-kD cut-off filter and fractionated on a C18 reverse phase column. Buffer A was 0.1% heptafluorobutyric acid (HFBA) and buffer B was 0.1% HFBA in acetonitrile. The gradient consisted of 100% buffer A (0 to 20 min), 0 to 12% buffer B (20 to 25 min), and 12 to 50% buffer B (25 to 80 min) at a flow rate of 200 μl/min. Sixty fractions of 200 μl each were collected from 20 to 80 min. (**B**) Fractions 28 and 29 from the separation shown in (A) were rechromatographed with the same acetonitrile gradient, but using trifluoroacetic acid (TFA) instead of HFBA as the organic modifier. For both panels, 3% of each peptide fraction was incubated with 1000 ^{51}Cr-labeled T2-B7 cells at room temperature for 2 hours. CTLs were then added at an effector to target ratio of 10 to 1 and further incubated at 37°C for 4 hours. Background lysis of T2-B7 by the CTL in the absence of any peptides was −3% in (A) and −4% in (B); positive control lysis of JY was 75% in (A) and 74% in (B). (**C**) Determination of candidate H-Y peptide by mass spectrometry combined with ^{51}Cr release assay. HPLC fraction 14 from the separation in Fig. 1B was chromatographed with an on-line microcapillary column effluent splitter as previously described (*11*, *13*). One-fifth of the effluent was deposited into 100 μl of culture media in microtiter plate wells for analysis with CTLs. The remaining four-fifths of the material were directed into the electrospray ionization source, and mass spectra of the peptides deposited in each well were recorded on a triple-quadruple mass spectrometer (Finnigan-MAT, San Jose, California). (◆), H-Y epitope reconstitution activity measured as percent specific lysis; (■), abundance of peptide 1171 measured as ion current at m/z 391.

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Cross-Presentation of Glycoprotein 96–associated Antigens on Major Histocompatibility Complex Class I Molecules Requires Receptor-mediated Endocytosis

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Abstract

Heat shock proteins (HSPs) like glycoprotein (gp)96 (glucose-regulated protein 94 [grp94]) are able to induce specific cytotoxic T lymphocyte (CTL) responses against cells from which they originate. Here, we demonstrate that for CTL activation by gp96-chaperoned peptides, specific receptor-mediated uptake of gp96 by antigen-presenting cells (APCs) is required. Moreover, we show that in both humans and mice, only professional APCs like dendritic cells (DCs), macrophages, and B cells, but not T cells, are able to bind gp96. The binding is saturable and can be inhibited using unlabeled gp96 molecules. Receptor binding by APCs leads to a rapid internalization of gp96, which colocalizes with endocytosed major histocompatibility complex (MHC) class I and class II molecules in endosomal compartments. Incubation of gp96 molecules isolated from cells expressing an adenovirus type 5 E1B epitope with the DC line D1 results in the activation of E1B-specific CTLs. This CTL activation can be specifically inhibited by the addition of irrelevant gp96 molecules not associated with E1B peptides. Our results demonstrate that only receptor-mediated endocytosis of gp96 molecules leads to MHC class I–restricted re-presentation of gp96-associated peptides and CTL activation; non–receptor-mediated, nonspecific endocytosis is not able to do so. Thus, we provide evidence on the mechanisms by which gp96 is participating in the cross-presentation of antigens from cellular origin.

Key words: heat shock protein–peptide complex • cross-priming • receptor-mediated endocytosis • cytotoxic T lymphocyte activation • dendritic cell

Introduction

Activation of CTLs with exogenous cell-associated antigens requires efficient uptake and presentation of these antigens by bone marrow–derived APCs. This phenomenon was first observed by Bevan (1–3) for the induction of CTLs against minor H antigens. Because the antigens were expressed in foreign donor cells with different MHC molecules, this process was termed "cross-priming." Since then, it has been shown that soluble protein antigens (4, 5), antigens expressed in MHC matched cells (6–8), or antigens encoded by naked DNA (9) also require uptake and re-presentation by MHC molecules expressed on the surface of professional APCs. Therefore, the term "cross-presentation" was introduced to describe the general re-presentation of exogenous cell-associated antigens by MHC class I (6) and MHC class II molecules (10). In addition to CTL activation, cross-presentation can also lead to the induction of CTL tolerance (11, 12).

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The nature of the APCs that are able to take up and re-present cell-associated antigens on MHC class I molecules remains elusive in vivo. However, in vitro studies suggest that dendritic cells (DCs)[1] (11–13), macrophages (14–16), or B cells (17) might be involved.

Several pathways for antigen uptake have been described, ranging from nonspecific mechanisms such as phagocytosis, pinocytosis, or macropinocytosis (18–23) to specific, receptor-operated mechanisms that include mannose- and scavenger-type receptors (22). Depending on the nature of the antigens, and consequently on the mode of uptake, antigens might be targeted to different processing compartments and be able to gain access to different antigen presentation pathways. CTL activation can be mediated by macropinocytosis or phagocytosis of exogenous soluble antigens (24–26). However, these pathways require high antigen concentrations and might therefore be of limited relevance in providing a mechanism for cross-presentation in vivo (23).

More recently, apoptotic bodies were shown to be phagocytosed by immature DCs, resulting in the activation of MHC class I–restricted T cells (13, 27). This uptake involves CD36 and the integrin receptor $\alpha_v\beta_5$ (28), which explains the high efficiency.

An additional pathway with potential relevance for cross-presentation became evident when the induction of tumor immunity and CTL activation through the injection of heat shock proteins (HSPs) such as glycoprotein (gp)96, HSP70, and HSP90 was discovered (for a review, see reference 29). The specificities of the CTL response were directed against the cells from which the HSPs were isolated. This can be explained by the association of the HSPs with peptides of cellular origin. Immune responses against several cellular antigens including minor H and tumor and viral antigens were induced (for a review, see reference 30) by using as little as 1–2 ng HSP–peptide complex in one particular case (31). It was postulated that the extremely efficient MHC presentation of HSP-associated peptides is accomplished by the receptor-mediated uptake of HSPs by professional APCs (32). Recently, binding of HSP70 and gp96 to a macrophage- and dendritic-like cell line was observed (33). This observation provides a possible explanation for the high immunogenic potential of HSPs in situations in which they are injected into mice or released from dying cells, in that they shuttle antigenic peptides to APCs (32). Receptor-mediated endocytosis of HSPs by professional APCs will lead to the accumulation of these peptide chaperones in cells crucially involved in the activation of CTLs.

We therefore decided to characterize the cell populations involved in receptor-mediated endocytosis of HSPs in detail, to follow the fate of endocytosed HSPs, and to test whether or not receptor-mediated endocytosis of HSPs indeed results in the re-presentation of HSP-associated peptides and subsequent activation of CTLs. The latter issue in particular is of crucial importance for the understanding of HSP-mediated cross-presentation, as antigen uptake by APCs does not necessarily correlate with the ability to cross-present antigens. Despite the fact that macrophages and DCs phagocytose apoptotic cells, only immature DCs are able to cross-present antigens and to activate CTLs (28).

Here, we demonstrate that members of the family of professional APCs such as macrophages, DCs, and B cells are able to bind the endoplasmic reticulum (ER)-resident HSP gp96 specifically. The binding was saturable and could be competed for with unlabeled gp96 molecules. The uptake of gp96 isolated from cells expressing the adenovirus type 5 (Ad5)-E1B epitope by the DC line D1 resulted in the activation of E1B-specific CTLs. More importantly, activation of Ad5-E1B–specific CTLs could be inhibited by competition with gp96 not associated with E1B peptide. This result clearly demonstrates that CTL activation is the consequence of receptor-mediated endocytosis of gp96 molecules followed by the class I–restricted re-presentation of associated peptides, and supports the participation of HSPs in cross-presentation of cell-associated antigens.

Materials and Methods

Mice, Cells, Antibodies, and Proteins. The DEC-205 knockout mice were provided by Michel Nussenzweig and Ralph Steinman (The Rockefeller University, New York, NY). BALB/c and C57BL/6 mice were obtained from Charles River Laboratories. MHC class II–deficient mice ABBN5 (34) and littermate ABBN6 were obtained from Taconic Farms. P388D1, RMA, and RMA-S mouse cell lines (American Type Culture Collection) were cultured in α-MEM. The cell line D2SC/1, representing an early progenitor of mouse splenic DC, and D1, a nontransformed, growth factor–dependent, long-term DC culture (35), were cultured in IMDM. All tissue culture media were supplemented with 10% FCS, 0.3 mg/ml L-glutamin, 100 U/ml penicillin/streptomycin, and 50 μM β-mercaptoethanol. To grow D1 cells, medium was additionally supplemented with 30% conditioned medium from the fibroblast cell line R1. Antibody to gp96 (SPA-850) was obtained from StressGen Biotechnologies. The following labeled antibodies to mouse and human antigens were obtained from BD PharMingen: H2-K^b–biotin, H2-A^b–biotin, CD8-FITC, IFN-γ-PE, CD16/CD32 (Fc block), CD45R/B220-PE, CD19-PE, CD14-PE, CD90.2 (Thy1.2)-PE, CD86 (B7.2)-PE, CD11c-PE, Mac-3–PE, CD1a-PE, CD83-PE, and IgG1-PE and IgG2a-PE isotype controls. Goat anti–rabbit-Alexa™ 546 and streptavidin-Alexa™ 546 (Molecular Probes) were used as secondary reagents. BSA, biotinylated BSA, OVA, and FITC were obtained from Sigma-Aldrich. Streptavidin-PE was purchased from Jackson Laboratories. BSA and OVA were labeled with FITC or biotin according to standard protocols. Free FITC molecules were removed by reaction with Tris and gel filtration through a Sephadex G-25 (Sigma-Aldrich) column. gp96 and gp96-FITC from the mouse cell line IGELa2 were provided by Immunosome. All animal studies were performed according to our institutional guidelines and approved by our Institutional Review Board.

Purification of gp96. The transporter associated with antigen processing (TAP)-deficient RMA-S SigE1B cell line has been generated by transfection of RMA-S with the adenovirus early

[1] *Abbreviations used in this paper:* Ad5, adenovirus type 5; BMDC, bone marrow–derived dendritic cell; DC, dendritic cell; ER, endoplasmic reticulum; gp, glycoprotein; HSP, heat shock protein; TAP, transporter associated with antigen processing.

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region 1 H2-D^b–restricted E1B epitope (VNIRNCCYI) targeted to the ER in a TAP-independent fashion (36). gp96 was purified from RMA, RMA-S, and RMA-S SigE1B cell lines as described (37). The approximate concentrations were determined by measuring the OD at 280 nm using an extinction coefficient of 1.0.

Cytometry (FACS®) Binding Assay. 10^5 cells were incubated for 30 min on ice in 100 μl IMDM and 10% FCS containing 30 μg/ml gp96-FITC or OVA-FITC, washed three times, and fixed in 1% paraformaldehyde. For competition experiments, a given excess of unlabeled gp96 was added together with 50 μg/ml gp96-FITC simultaneously. For staining of mouse spleen cells (including erythrocytes) and human PBLs, PE-conjugated antibodies were added as markers for different cell types. Immature DCs were prepared from bone marrow of C57BL/6 mice (38) and human blood monocytes (39) as described. Cytometry measurements were performed on a FACSCalibur™ (Becton Dickinson).

Internalization Studies in Confocal Microscopy. Immature bone marrow–derived DCs (BMDCs) were prepared from C57BL/6 mice as described (38). On day 6 of their preparation, the BMDCs were tested for CD11c, CD86, and MHC class II expression and were seeded on cover slips, precooled, and incubated for 30 min on ice with IMDM containing 10% FCS and 50 μg/ml gp96-FITC ("pulse"). The coverslips were washed twice and incubated in IMDM medium for 15 min or longer at 37°C ("chase"), washed, and fixed in 3.7% paraformaldehyde in PBS. For the colocalization experiments, cells were pre-incubated with Fc block (α-CD16/CD32) followed by biotinylated antibodies to H2-K^b or H2-A^b and 50 μg/ml gp96-FITC together with streptavidin-Alexa™ 546. For staining of lysosomes, cells were fixed with methanol/aceton (1:1, −20°C) and incubated with α–Lamp-1 (provided by M. Fukuda, La Jolla Research Center, La Jolla, CA) and goat anti–rabbit–Alexa™ 546. For microscopy, a ZEISS LSM 510 laser scanning microscope was used. "Bleeding" of emission into other detection channels was excluded using the multitracking modus of the LSM 510. Thickness of the optical plane was adjusted by the pinhole to be <1 μm.

Immunization of Mice with gp96. C57BL/6 mice were immunized intraperitoneally with 30 μg gp96 purified from RMA-S SigE1B cells. After 10 d, mice were killed and the spleen cells were restimulated with E1B-expressing XC3 cells or Ad5-E1B peptide (50 ng/ml). Specific lysis of RMA-S SigE1B cells by CTLs contained in the spleen culture was determined by a standard chromium release assay 5 d after restimulation and after a second restimulation with XC3 cells or Ad5-E1B peptide (50 ng/ml).

CTL Cross-Presentation Assay. The CTL clones 100B6, 0.1C2, and LN5 were described previously (36, 40). CTL clones were restimulated on a weekly basis by incubation with the Ad5-E1B/E1A–expressing tumor cell line XC3. The E1B peptide was synthesized on a ABI 432 A peptide snythesizer (Applied Biosystems) applying Fmoc strategy.

Activation of CTL clones was assessed by measurement of intracellular IFN-γ production. 2.5×10^4 D1 cells were incubated with 20 μg/ml gp96 purified from RMA-S SigE1B, RMA, or RMA-S cells for 2 h at 37°C. For competition experiments, an excess of gp96 from RMA or RMA-S was added, washed four times, and incubated with 2.5×10^5 CTLs for 12 h at 37°C. 10 μg/ml Brefeldin A was added for an additional 5 h at 37°C. Cells were washed, fixed, and perforated with saponin. The fixed cells were stained with PE-labeled anti–IFN-γ or isotype control and FITC-labeled anti-CD8 antibodies, and were measured by flow cytometry.

Results

gp96 Binds Specifically to APC Lines. Recent experiments demonstrated that HSPs are able to interact specifically with a macrophage- and a DC-like cell line (33). Therefore, we further characterized the cell types able to interact with gp96 in a specific manner. For this purpose, we incubated several cell lines with FITC-labeled gp96, always at 4°C to exclude endocytosis. We only observed a specific interaction of gp96 with APC lines like P388D1, D2SC/1, and D1, but not with the lymphoina cell lines RMA, EG.7, and T1 (Fig. 1, A and B). Increasing the total concentration of gp96-FITC, the binding displayed saturation at a total concentration of 30 μg/ml (Fig. 1 C) and could only be competed for by unlabeled gp96, but not by OVA (Fig. 1 A) or BSA (not shown). A onefold excess of

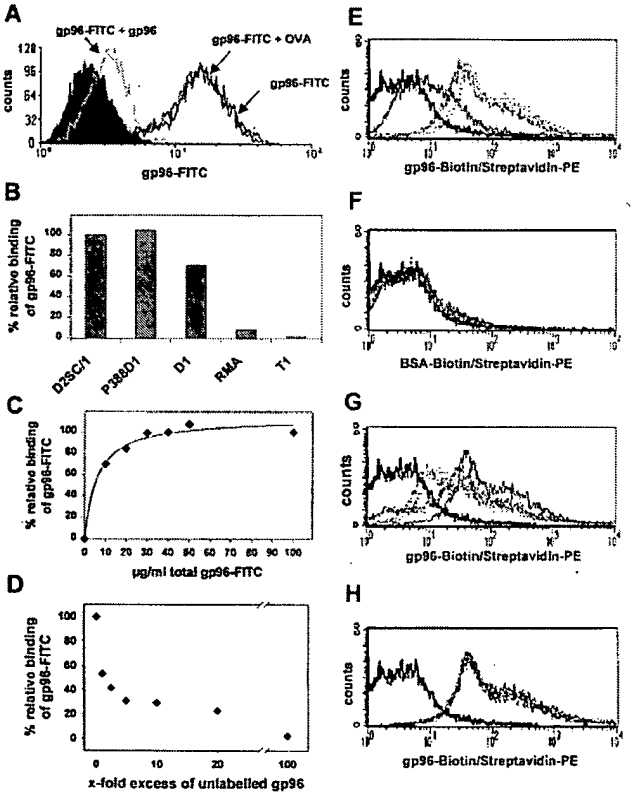

Figure 1. Specific binding of gp96-FITC to APC cell lines and BMDCs. Binding of 3 μg gp96-FITC to 10^5 D2SC/1 cells was performed, always at 4°C, in 100 μl IMDM containing 10% FCS. This binding could be competed by a 10-fold excess of unlabeled gp96, but not OVA. (A) Specific binding of gp96-FITC was observed on D2SC/1 (DC progenitor), P388D1 (macrophage), and D1 (DC), but not on RMA and T1 cells. (B) Binding could be saturated at ~30 μg/ml for 10^5 D2SC/1 cells (C) and competed almost completely by a 100-fold excess of unlabeled gp96. (D) Binding is given as relative values, where 100% represents maximum binding of gp96-FITC. The concentration values shown give total concentration of gp96-FITC added to the cells. (E) Binding of 1 μg (bold line), 5 μg (broken line), and 10 μg (dotted line) gp96-biotin/streptavidin-PE to immature BMDCs from C57BL/6 mice. (F) No binding was observed for BSA-biotin/streptavidin-PE. Binding of 10 μg gp96-biotin (bold line) to BMDC is competed in a similar fashion to D by unlabeled gp96 (G), but not by unlabeled BSA (H).

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unlabeled gp96 resulted in a 50% reduction, and a fivefold excess resulted in an ~75% reduction of gp96-FITC binding at saturation point, which could be inhibited completely using an excess of up to a 100-fold (Fig. 1 D). These data correspond to the theoretical values of 50% and 83% (1:1 and 1:5 dilution of gp96-FITC with unlabeled gp96), demonstrating that the FITC labeling of gp96 did not significantly affect the binding characteristics to its putative receptor. No inhibition was observed using an excess of up to 400-fold of OVA or BSA (data not shown). These data demonstrate the presence of a specific gp96 receptor that is expressed on APCs but not on other cell lines (Fig. 1, A–D).

gp96 Interacts Specifically with Primary APCs in Mice and Humans. More importantly, gp96 (Fig. 1 E) but not BSA (Fig. 1 F) bound efficiently to immature BMDCs prepared (38) from C57BL/6 mice, and could be competed for by increasing amounts of unlabeled gp96 (Fig. 1 G) but not by BSA (Fig. 1 H). Specific binding was also observed when mouse spleen cells from BALB/c mice were incubated with gp96-FITC (Fig. 2). gp96 interacted specifically with cells that stained positive for MHC class II and CD45 (B220), but not with cells positive for CD90 (Thy-1) molecules. Setting the forward and side scatter gate on the bigger cells, including cells of the myeloid lineage, CD11c– and Mac-3–positive cells were also positive for gp96-FITC, indicating that the expression of the gp96 receptor is restricted to professional APCs. No staining was observed using OVA-FITC (Fig. 2, left) or BSA-FITC (data not shown). The identical outcome was observed using spleen cells from C57BL/6 mice (not shown). A similar gp96-FITC staining pattern was obtained for human PBLs. HLA-DR–, CD86-, CD19-, and CD14-positive but not CD2- or CD3-positive cells interacted specifically with gp96-FITC (Fig. 3 A). Again, no staining was observed using OVA-FITC. gp96 binding to monocytes was slightly better than to B cells in human PBLs. As expected, DCs expressing CD1a and CD83 were not detected. To determine gp96-FITC binding to this cell type, we differentiated DCs from human PBLs by the application of GM-CSF and IL-4. The whole DC population generated stained positive with gp96 but not BSA (Fig. 3 B).

DEC-205 and MHC Class II Molecules Are Not Required for gp96 Binding. Because gp96 molecules contain a single high-mannose oligosaccharide (41, 42), we addressed the question of whether this might allow the uptake by the DEC-205 receptor. DEC-205 is expressed on DCs and thymic epithelial cells and is capable of directing captured soluble exogenous antigens to a specialized antigen processing compartment (43). DCs were prepared from bone marrow of wild-type and DEC-205$^{-/-}$ mice (provided by Michel Nussenzweig and Ralph Steinman) and incubated with increasing amounts of gp96-FITC. FACS® analysis revealed identical staining (Fig. 4 A), suggesting that the DEC-205 receptor is not involved in the binding of gp96 molecules by DCs.

We further speculated whether MHC class II might function as a receptor for gp96 because gp96 showed binding to all MHC class II–positive mouse spleen cells and human PBLs. Binding together with marker antibodies to spleen cells from MHC class II knockout mice and their littermates did not reveal any difference (shown for MHC class II antibody in Fig. 4 B; other markers not shown), indicating that MHC class II molecules do not function as gp96 receptors.

gp96 is Endocytosed Efficiently and Colocalizes with Recycled MHC Class I and Class II Molecules. Recently it has been suggested that gp96-FITC bound to peritoneal macrophages is endocytosed into early endosomes but does not reach the stage of lysosomes (44). We also attempted to determine the faith of receptor-bound gp96 at the cell surface of APCs by confocal microscopy (Fig. 5) using authentic DCs (BMDCs from C57BL/6 mice). Initial binding of

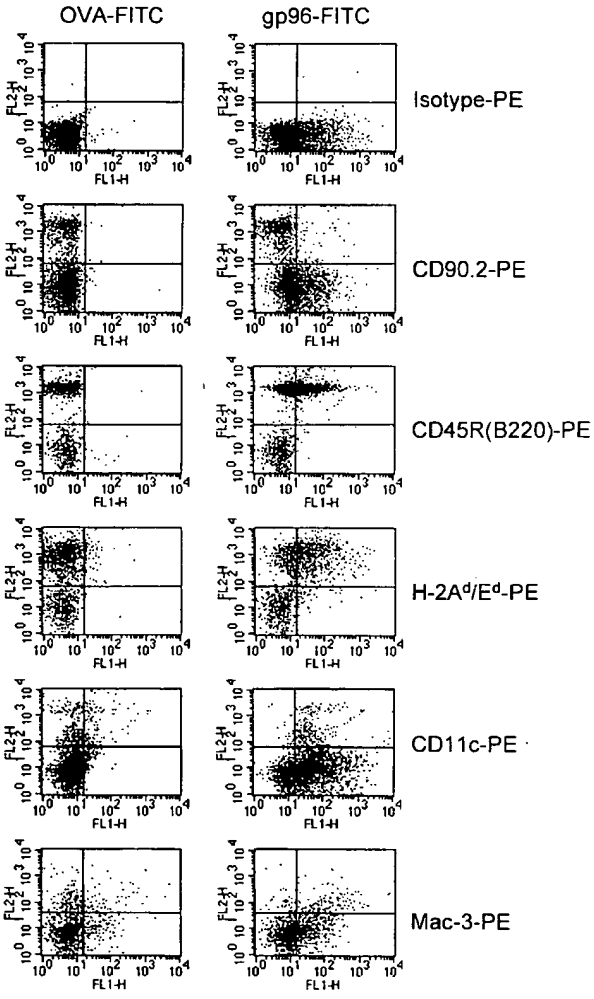

Figure 2. Specific binding of gp96-FITC to B cells, macrophages, and DCs, but not to T cells of a spleen cell culture. 10^5 fresh BALB/c spleen cells were stained with 5 μg OVA-FITC (left) or gp96-FITC (right), and different PE-labeled cell type marker antibodies to CD90.2 (Thy-1.2, T cells), CD45R/B220 (B cells), I-A^d/E^d, CD11c (DCs), and Mac-3 (monocytes and macrophages). Macrophages and DCs were counted in a different gate,than lymphocytes with a higher forward scatter value.

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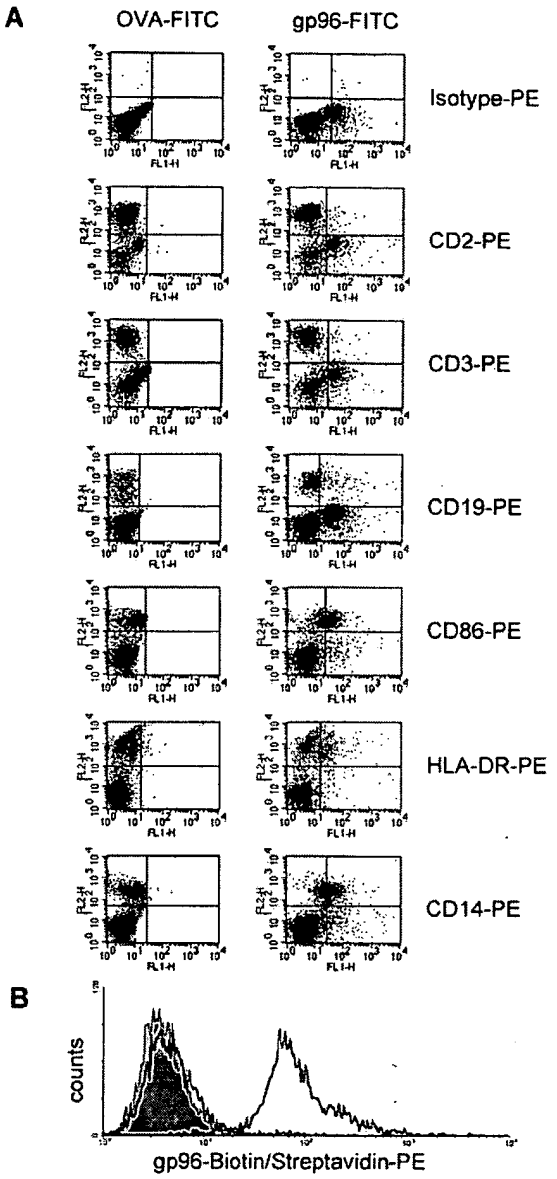

Figure 3. gp96-FITC binds to APCs in human PBL culture, but not to T cells. (A) 10^5 fresh human PBLs were stained with 5 μg OVA-FITC (left) or gp96-FITC (right) together with PE-labeled antibodies to the following cell surface antigens: CD2 (T and NK cells), CD3 (T cells), CD19 (B cells), CD86, HLA-DR, and CD14 (monocytes). The gate was set on all living cells. Therefore monocytes appear as a population with a slightly higher autofluorescence than lymphocytes in both fluorescence channels. Comparing the shifts of each population, monocytes showed slightly better binding of gp96 than B cells. (B) Binding of 0 μg (gray shaded) and 5 μg (bold line) gp96-biotin to immature DCs prepared from human PBLs. 10 μg of BSA-biotin (bold line) did not display binding.

FITC-labeled gp96 to the cell surface at 4°C to prevent endocytosis revealed a patched pattern. Further incubation at 37°C led to efficient endocytosis of gp96. Colocalization with lysosomes labeled with Lamp-1 antibody (45) was not observed after 15, 30, 45, 60, and 90 min of endocytosis (shown for 60 min in Fig. 5). Recently, it has been reported that internalized cell surface MHC class I molecules, like class II molecules, are able to bind their antigen in endosomal compartments, suggesting these vesicles to be putative MHC class I and class II loading compartments for exogenously derived antigen (46). We therefore attempted to determine whether gp96 taken up by receptor-mediated endocytosis can be found in compartments containing recycled MHC class I and class II molecules. Indeed, after 15 min of endocytosis, nearly all of the endocytosed H2-K^b and H2-A^b molecules colocalized with gp96. Similar results were obtained using gp96-FITC bound to the cell surface of the D2SC/1 cell line, where after 15 min of endocytosis, gp96 colocalized with transferrin Texas red (as marker for early endosomes) and endocytosed H2-K^d molecules, but were excluded from lysosomes after 30 min (data not shown).

gp96-associated Peptides Are Loaded onto MHC Class I Molecules as a Result of Receptor-mediated Endocytosis. gp96 molecules have been observed to enter APCs by receptor-mediated endocytosis as well as by non–receptor-mediated, nonspecific endocytosis or macropinocytosis (33, 44). The latter nonspecific pathways have been described many times before to introduce exogenous proteins into the MHC class I–restricted antigen pathway, but unlike receptor-mediated endocytosis require high concentrations of antigens (for a review, see reference 22).

To investigate whether receptor-mediated endocytosis can lead to cross-presentation of gp96-associated antigens, we have isolated gp96 from RMA-S SigE1B cells that stably express the H2-D^b–restricted E1B epitope of Ad5, fused with an ER-targeting signal sequence. C57BL/6

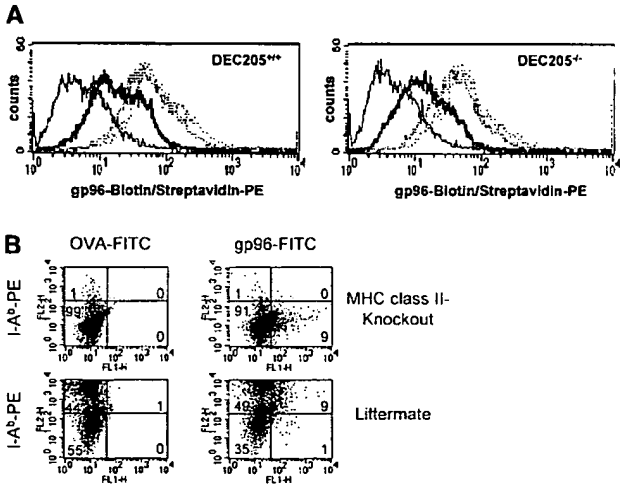

Figure 4. DEC-205 and MHC class II do not function as receptors for gp96. Binding of gp96-biotin to BMDCs from wild-type and DEC-$205^{-/-}$ mice (A) as well as binding of gp96-FITC to spleen cells from MHC class $II^{-/-}$ mice and their littermate (B) showed identical staining. For staining of spleen cells in B, different cell surface markers were used (shown in Fig. 2). Only antibody to MHC class II is shown.

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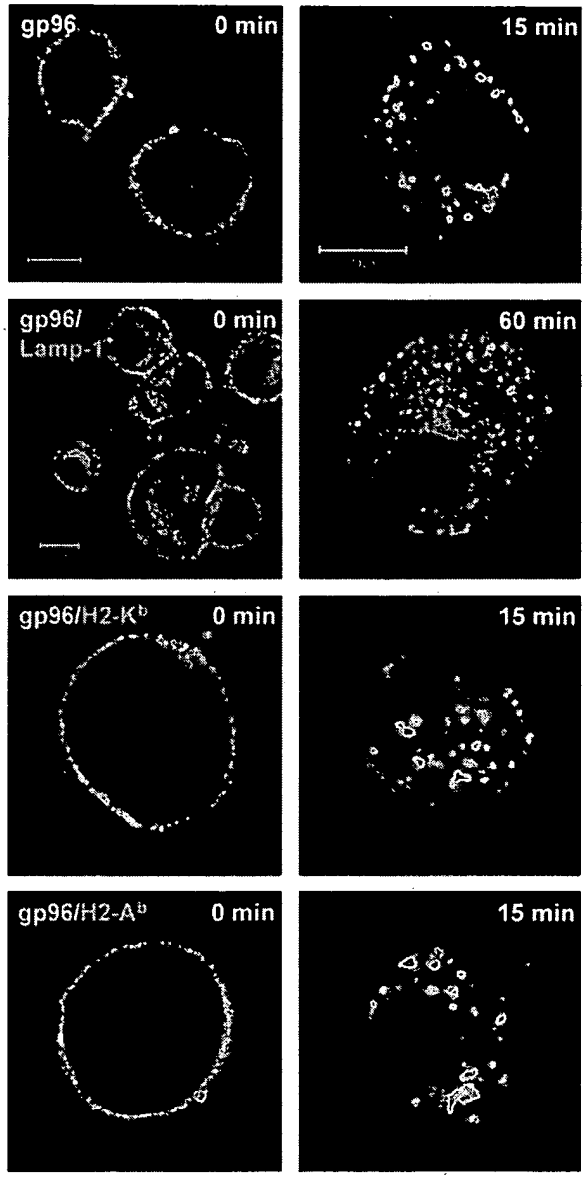

Figure 5. gp96-FITC is endocytosed by BMDCs efficiently and colocalizes with endocytosed MHC class I and class II molecules, but does not target to lysosomes. Internalization of gp96-FITC was followed by confocal microscopy. Representative sections are displayed. Coverslip-grown BMDCs were incubated with 50 μg/ml gp96-FITC (shown in false color green) on ice, washed, chased for 15 min or longer at 37°C, and fixed in paraformaldehyde. To follow the fate of gp96-FITC after 15, 30, 45, 60, and 90 min (only 60 min is shown) of endocytosis, cells were fixed and permeabilized with methanol/aceton and stained with antibody to the Lamp-1 and secondary Alexa™ 546-coupled antibody to visualize lysosomes (shown in false color red). No colocalization of gp96 and Lamp-1 was observed. Furthermore, cells were stained with biotinylated antibodies to MHC class I (H2-K^b) and class II (H2-A^b) and secondary streptavidin-Alexa™ 546 (both shown in red) as well as gp96-FITC (green) on ice, washed, and chased at 37°C for 15 min. After 15 min of endocytosis, nearly all vesicles containing endocytosed gp96 and MHC class I and class II molecules colocalize (shown in yellow as result of overlapping green and red).

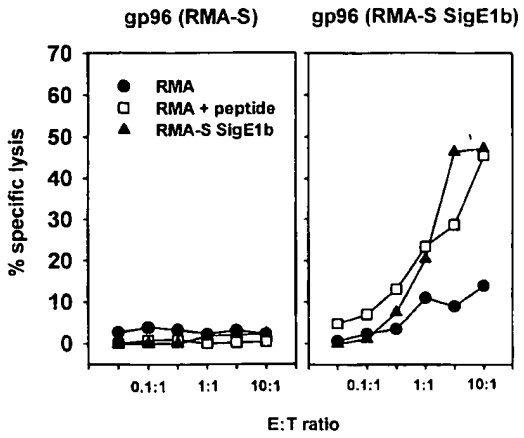

Figure 6. gp96–E1B complexes generate a CTL response in vivo. gp96 was purified from RMA-S SigE1B and RMA-S cells. 30 μg of gp96 from either cell type was injected into C57BL/6 mice intraperitoneally. The specificity of the generated CTLs was assayed by ^{51}Cr-release of RMA-S SigE1B cells (▲), RMA cells incubated with 100 ng/ml Ad5-E1B peptide (□), or RMA cells (●). The figure shows one representative of three independent experiments.

mice immunized with these gp96 molecules generated CTLs that recognized efficiently RMA-S SigE1B and RMA cells pulsed with the Ad5-E1B peptide, but not RMA cells, demonstrating the presence of the Ad5-E1B epitope on gp96 molecules. Immunization with control gp96 molecules from RMA-S cells did not induce Ad5-E1B–specific CTL responses (Fig. 6).

To test whether the E1B epitope attached to gp96 was re-presented to CTLs after uptake by APCs, gp96 isolated from RMA-S SigE1B (or control gp96 from RMA-S cells) was incubated with the DC cell line D1 for 2 h at 37°C. The D1 cells were further incubated overnight with the Ad5-E1B–specific CTL clones 100B6 and 0.1C2 or control CTL clone LN5, specific for the Ad5-E1A epitope. Intracellular IFN-γ production was measured to determine CTL activation via the re-presentation of the Ad5-E1B peptide. As shown in Fig. 7 A, incubation of 0.1C2 CTLs with D1 cells pulsed with RMA-S SigE1B gp96 resulted in the activation of T cells. This activation was not observed if control gp96 isolated from RMA-S cells was used or if gp96 isolated from RMA-S SigE1B cells was incubated with the CTLs in the absence of D1 cells. The latter experiment clearly demonstrates that D1 cells, which efficiently bind gp96 molecules (shown in Fig. 1 B), are required for the re-presentation. The T cells themselves are not able to bind gp96 (shown in Figs. 2 and 3), and consequently do not stimulate each other.

Most importantly, however, the activation of Ad5-E1B–specific CTLs by gp96 from RMA-S SigE1B cells could be inhibited by the addition of a twofold excess of irrelevant gp96 molecules from RMA-S and RMA cells. This excess of gp96 was able to reduce the binding of gp96-FITC by 60% (Fig. 1 D), and eliminated the activation of 0.1C2 CTLs by gp96 molecules from RMA-S SigE1B cells almost

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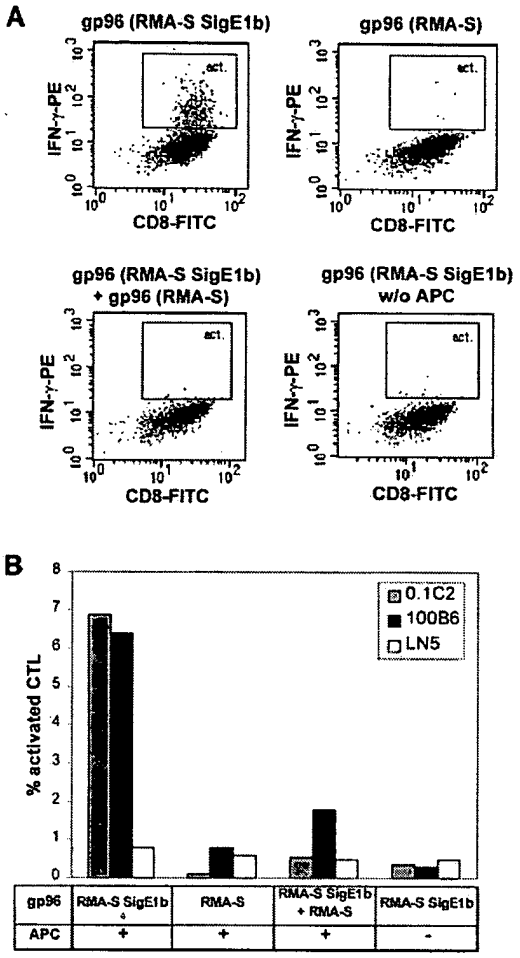

Figure 7. Specific activation of CTLs by DC-mediated cross-presentation of gp96-associated antigen requires receptor-mediated endocytosis of gp96–antigen complexes. (A) Activation of Ad5-E1B–specific CTL clone 0.1C2 was assayed by intracellular IFN-γ staining in flow cytometry. D1 DCs as APCs could activate the CTLs after prior incubation of D1 with gp96–E1B complexes purified from RMA-S SigE1B (top left), but not with irrelevant gp96 isolated from RMA-S (top right) or RMA cells (data not shown) or in the absence of D1 cells (bottom right). Moreover, activation by gp96–E1B complexes could be competed with a twofold excess of gp96 from RMA-S (bottom left) or RMA (not shown), but not with the same excess of BSA (not shown), indicating the presence of a receptor-mediated pathway responsible for processing of gp96 by D1 cells. PE-labeled isotype control antibody was always negative (data not shown). Results are representative for at least three experiments. (B) Summary of the activation of Ad5-E1B–specific CTL clones 0.1C2 and 100B6 as well as control CTL clone LN5 specific for Ad5 E1A. Graph shows the percentage of activated CTLs present in the gate shown in A. Addition of Ad5–E1B peptide to D1 cells resulted in the activation of ~25% of CTL clones 0.1C2 and 100B6 (data not shown).

completely. The identical scenario was observed for a different Ad5-E1B–specific CTL clone, 100B6, but not for LN5 CTLs, which are specific for the control Ad5-E1A CTL epitope (Fig. 7 B). No competition was observed by using a twofold excess of BSA as control (data not shown).

Discussion

HSPs have been shown previously to induce specific immune responses against tumor, minor H, and viral antigens (for reviews, see references 29 and 30). This feature is based on peptides that are associated with HSPs and on the fact that HSPs, by an unknown mechanism, can interact very efficiently with APCs to result in the re-presentation of HSP-associated peptides and subsequent activation of T cells (31, 47). We have now shown in this study that specific binding of low amounts of gp96 to a receptor present on mouse and human professional APCs is indeed required for the MHC class I–restricted re-presentation of gp96-associated peptides.

The nature of the gp96 receptor still remains unclear. We reported earlier that gp96 binding to the macrophage line P388D1 cannot be inhibited by mannan, thus arguing against the participation of the mannose receptor. Using DCs from DEC-205$^{-/-}$ mice, we show here that this receptor as well, which displays strong homology to the mannose receptor present on macrophages (43), is unlikely to be involved, because gp96-FITC binding is indistinguishable from that observed for DCs of wild-type mice (Fig. 4 A). Because DnaK and HSP73 molecules have been reported to bind to certain allelic products of MHC class II (48, 49), they could represent another potential receptor for HSPs on the surface of APCs. The observation that gp96 binds to all MHC class II–positive cells could indicate that gp96 also uses MHC class II molecules as a receptor. However, anti–MHC class II antibodies were not able to inhibit the binding of gp96-FITC molecules (data not shown), and cells from MHC class II$^{-/-}$ mice showed identical gp96 binding compared with wild-type mice, thus arguing against MHC class II molecules being the receptor for gp96 (Fig. 4 B).

We further demonstrate in this study that the specific interaction of gp96 molecules with DCs results in re-presentation of associated peptides and specific activation of CTLs. gp96 molecules isolated from RMA-S SigE1B cells that carry the Ad5-E1B CTL epitope (Fig. 6) are able to activate Ad5-E1B–specific CTLs after incubation with the DC line D1, as visualized by intracellular IFN-γ staining. The control CTL line LN5 is not activated by any of the gp96 preparations tested (Fig. 7). More importantly, we are able to show here for the first time that receptor-mediated endocytosis of gp96 is indeed required for the re-presentation and subsequent activation of CTLs. By inhibiting the specific binding of RMA-S SigE1B–derived gp96 with a twofold excess of unrelated gp96 molecules that have been shown to reduce gp96-FITC binding by 60% (Fig. 1 D), we completely abolish the activation of Ad5-E1B–specific CTLs (Fig. 5). This low excess of unrelated gp96 was chosen on purpose to exclude potential toxic effects of a high gp96 concentration. Using synthetic E1B peptide, ~25% of CTLs could be activated (data not shown), compared with 6–7% activated CTLs, as shown in Fig. 7. Therefore, the amount of RMA-S SigE1B gp96 was not able to activate all possible CTLs, most likely because of limiting

amounts of peptide. As the activation of CTLs requires the activating signal to be above a certain threshold, the amount of antigen presented by MHC class I molecules in the presence of competitor could easily be below this threshold, explaining the lack of a CTL response with a twofold excess of irrelevant gp96 not associated with E1B peptide.

Because only receptor-mediated endocytosis of labeled gp96 but not nonspecific, non–receptor-mediated uptake such as pinocytosis or macropinocytosis can be inhibited by an excess of unlabeled gp96 (33), our results clearly demonstrate that receptor-mediated endocytosis of gp96 molecules is the cellular pathway responsible for re-presentation of gp96-associated peptides by MHC class I molecules. Therefore, our results provide evidence for the hypothesis that professional APCs possess receptors that are able to interact specifically with HSPs (32) and direct HSP-associated peptides into the MHC class I–restricted antigen presentation pathway. This now explains why very small amounts of gp96–peptide complexes can activate T cells.

The exact intracellular pathway for the re-presentation of gp96-associated peptides requires further clarification. Confocal microscopy data point in the direction that gp96 heads for early endosomes but does not enter lysosomes. We could show that gp96 after receptor-mediated uptake enters compartments containing MHC class I and class II molecules. It can be speculated that these compartments function as putative loading compartments where antigen could be transferred to MHC class I and class II molecules (46), but it cannot be excluded that gp96–antigen complexes enter the cytosol specifically, as recently suggested for immunoglobulin–antigen complexes after endocytosis by Fc receptors in DCs (50).

Further identification of the pathway responsible for the re-presentation of gp96-associated peptides will also contribute to the understanding of the phenomenon termed cross-presentation. Until now, cross-presentation of MHC class I–restricted antigens has been shown to be induced by receptor-mediated phagocytosis of apoptotic bodies (27, 28), exosomes (51), bacteria (52), and proteins, either denatured or immobilized (26) by phagocytic or nonphagocytic mechanisms (22). Unlike the latter two pathways, which in most require cases high concentrations of the antigens, receptor-mediated endocytosis of HSPs operates efficiently at antigen concentrations of ~1–2 ng per mouse (31), and might be as efficient as receptor-mediated phagocytosis of apoptotic cells or receptor-mediated endocytosis of proteins by surface immunoglobulins on B cells. One can envisage that HSPs, released from dying cells, bind to HSP receptors of professional APCs and are endocytosed before the associated peptides are re-presented by MHC class I molecules.

The antigen carriers in apoptotic cells or exosomes are unknown, but one interesting possibility is that HSPs chaperone the antigenic peptides, thus protecting them from further degradation and directing them to the correct intracellular loading compartment. In line with this speculation is the observation that HSP70 is one of the proteins found in close association with the transferrin receptor in exosomes derived from reticulocytes (53). Whether or not the induction of apoptosis leads to a general increase of HSPs is still controversial and might depend on factors that are still to be determined. For tumor cells, it was reported that apoptotic death was associated with low HSP expression levels (54), whereas for PMNs, increased apoptosis coincided with induction of Hsp72 (55). Nevertheless, an increase of HSP expression levels generally seems to correlate with increased immunogenicity (54, 56), supporting the above-mentioned hypothesis.

The finding that cells deficient in TAP are still able to cross-prime as efficiently as wild-type cells (57) does not contradict the involvement of HSPs in cross-presentation. It shows that the ER-resident HSP gp96 alone is not essential for cross-priming, but it also does not exclude the participation of other HSPs such as HSP70 or HSP90 that might compensate for the absence of immunogenic gp96–peptide complexes. Another argument formulated against the participation of HSPs in cross-presentation of cellular antigens is based on an experiment performed by Carbone and Bevan (58), in which splenocytes were incubated with OVA or β-galactosidase, washed, and injected into mice. Because of the nonspecific coating of cells with the soluble proteins, an association with HSPs might be difficult to imagine. However, the incubation conditions (37°C, 10 mg/ml protein, 10 min), do not exclude the uptake and processing of proteins and the subsequent loading of antigenic peptides onto HSPs. In addition, several different pathways for cross-presentation, including apoptotic cells, exosomes, and receptor-mediated endocytosis of HSPs, might exist in parallel, each one able to induce the cross-presentation of different types of antigens.

More detailed knowledge about the gp96 receptor, its intracellular transport, and the regulation of expression in different cell types will deepen our understanding of the role of gp96 and possibly HSPs in general in cross-presentation, and could greatly improve the application of gp96 for the induction of specific immune responses in vivo.

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HSP90alpha and HSP90beta isoforms selectively modulate MHC class II antigen presentation in B cells.

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Two isoforms of heat shock protein (HSP) 90, alpha and beta, are abundantly expressed in the cytoplasm of cells, yet only HSP90alpha serves as a chaperone to potentiate epitope presentation in the context of MHC class I molecules. By contrast, the role of HSP90 isoforms in MHC class II presentation of exogenous and endogenous Ags remains less clear. Studies here using human B lymphoblasts demonstrate the importance of HSP90alpha and HSP90beta isoforms in selectively regulating class II presentation of the diabetes autoantigen glutamic acid decarboxylase (GAD). Inactivation of HSP90 function using geldanamycin or radicicol inhibited MHC class II presentation of exogenous and endogenous GAD, but did not perturb the presentation of several other intra- and extracellular Ags. Treatment of human B cells with geldanamycin and radicicol did not alter cellular MHC class II expression, but did induce a stress response in these APCs. Yet, cell stress alone failed to perturb MHC class II presentation of GAD. HSP90 was found to associate with select Ags such as GAD in cells and ex vivo. Knockdown of HSP90alpha or HSP90beta expression using small interfering RNA decreased the abundance of each isoform, respectively, but did not affect MHC class II expression or induce a stress response. Notably, disruption of HSP90alpha or HSP90beta expression specifically inhibited class II presentation of the exogenous and endogenous GAD Ag. Precomplexing HSP90 with GAD Ag enhanced exogenous GAD Ag presentation. These results demonstrate a requirement for HSP90alpha and HSP90beta in regulating class II presentation of select Ags.

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Bacterial Heat Shock Proteins Enhance Class II MHC Antigen Processing and Presentation of Chaperoned Peptides to CD4$^+$ T Cells[1]

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APCs process heat shock protein (HSP):peptide complexes to present HSP-chaperoned peptides on class I MHC molecules, but the ability of HSPs to contribute chaperoned peptides for class II MHC (MHC-II) Ag processing and presentation is unclear. Our studies revealed that exogenous bacterial HSPs (*Escherichia coli* DnaK and *Mycobacterium tuberculosis* HSP70) delivered an extended OVA peptide for processing and MHC-II presentation, as detected by T hybridoma cells. Bacterial HSPs enhanced MHC-II presentation only if peptide was complexed to the HSP, suggesting that the key HSP function was enhanced delivery or processing of chaperoned peptide Ag rather than generalized enhancement of APC function. HSP-enhanced processing was intact in MyD88 knockout cells, which lack most TLR signaling, further suggesting the effect was not due to TLR-induced induction of accessory molecules. Bacterial HSPs enhanced uptake of peptide, which may contribute to increased MHC-II presentation. In addition, HSPs enhanced binding of peptide to MHC-II molecules at pH 5.0 (the pH of vacuolar compartments), but not at pH 7.4, indicating another mechanism for enhancement of MHC-II Ag processing. Bacterial HSPs are a potential source of microbial peptide Ags during phagocytic processing of bacteria during infection and could potentially be incorporated in vaccines to enhance presentation of peptides to CD4$^+$ T cells. *The Journal of Immunology*, 2004, 173: 5130–5137.

Heat shock proteins (HSPs)[4] are molecular chaperones expressed by prokaryotes and eukaryotes that bind polypeptide chains, prevent aggregation, and support protein folding (1). Expression of many HSPs is increased with stress (e.g., heat, anoxia, glucose starvation). Members of the HSP70 family are constitutively expressed in eukaryotic and prokaryotic cells. This family includes *Escherichia coli* DnaK and *Mycobacterium tuberculosis* (MTB) HSP70. These HSPs bind hydrophobic regions of nascent polypeptides and unfold or disaggregate misfolded proteins to yield productive folding intermediates.

Mammalian HSPs also have immunological functions (2, 3). Mammalian HSP70 (4–7), HSP90 (5, 8), gp96 (5, 8–12), calreticulin (13, 14), and HSP110 (15) bind peptides to form highly immunogenic HSP:peptide complexes that are processed by macrophages and dendritic cells, resulting in presentation of HSP-chaperoned peptides by class I MHC (MHC-I) molecules to activate CD8$^+$ T cells (16). Immunization with HSPs purified from tumor cells protects mice from subsequent challenge with the same tumor through activation of CD8$^+$ T cells (2, 4, 5, 11, 13, 15). The enhanced immunogenicity conferred by mammalian HSPs requires binding of antigenic peptide to the HSP (4), and the immunogenicity of empty HSPs can be reconstituted by loading them with peptides (17). Our recent studies have established that bacterial HSPs, e.g., *E. coli* DnaK and MTB HSP70, are also processed by macrophages and dendritic cells for MHC-I presentation of chaperoned peptides (18).

Almost all studies of immunogenicity of exogenous HSP:peptide complexes have assessed MHC-I Ag presentation and CD8$^+$ cell responses. In contrast, little research has been performed to examine the ability of exogenous HSPs to deliver chaperoned peptides for enhanced class II MHC (MHC-II) Ag processing and presentation to activate CD4$^+$ T cells. Heat shock enhances MHC-II Ag processing, and endogenous HSPs expressed by APCs have been proposed to contribute to MHC-II Ag processing (19–22). We are aware of only a single study relating to the ability of exogenous HSP:peptide complexes to induce CD4$^+$ T cell responses. This study demonstrated that peptide bound to MTB HSP70 enhanced delayed-type hypersensitivity (23). Specific MHC-II Ag-processing functions, however, have not been attributed to HSPs, and the ability of HSPs, either mammalian or bacterial, to promote MHC-II processing and presentation of chaperoned peptides is essentially unexplored.

Peptides naturally associated with mammalian HSPs include self peptides, tumor peptides, or viral peptides, because these are derived from proteins synthesized in mammalian cells. In contrast, peptides naturally associated with bacterial HSPs include bacterial Ags, suggesting that bacterial HSPs may contribute to antibacterial CD4$^+$ T cell responses or could be used therapeutically to generate such responses. These mechanisms could be especially important to augment host immunity to intracellular bacterial pathogens (e.g., MTB, *Mycobacterium leprae*, and *Salmonella*), because CD4$^+$ T cells are important in the control of intracellular bacterial pathogens either directly as effector cells or as helper cells for CD8$^+$ T cells.

This study reveals that two bacterial HSPs, *E. coli* DnaK and MTB HSP70, are capable of delivering an extended synthetic peptide for enhanced processing and MHC-II presentation of a constituent epitope. Thus, bacterial HSPs promote MHC-II Ag

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[4] Abbreviations used in this paper: HSP, heat shock protein; MHC-I, class I MHC; MHC-II, class II MHC; MFV, mean fluorescence value; MTB, *Mycobacterium tuberculosis*.

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processing and presentation of chaperoned peptide. We also demonstrate that HSPs increase uptake of peptide and enhance binding of peptide to MHC-II molecules at acidic pH (as found in endosomes and phagosomes). We propose that bacterial HSPs may deliver HSP-bound bacterial peptides during phagocytic processing of bacteria, thereby promoting MHC-II presentation of bacterial Ags and $CD4^+$ T cell responses during infection with bacterial pathogens. Furthermore, this mechanism could provide a basis for use of bacterial HSPs in vaccines to enhance priming of $CD4^+$ T cell responses.

Materials and Methods

Cells and medium

Unless otherwise specified, incubations were at 37°C and 5% CO_2 in standard medium containing DMEM (Invitrogen Life Technologies, Carlsbad, CA), 10% heat-inactivated FCS (HyClone, Logan, UT), 50 μM 2-ME (BioRad, Hercules, CA), 1 mM sodium pyruvate (Invitrogen Life Technologies), 10 mM HEPES buffer (Invitrogen Life Technologies), and antibiotics. B6D2F1/J and C57BL/6 female mice were from The Jackson Laboratory (Bar Harbor, ME). H2-$DM^{-/-}$ mice (24) were generously provide by L. Van Kaer (Vanderbilt University, Nashville, TN). $MyD88^{-/-}$ mice were generously provided by O. Takeuchi and S. Akira (Osaka University, Osaka, Japan) (25) and bred onto C57BL/6 background for five to seven generations. B6D2F1/J mice were used for all experiments, except those involving knockout models, which used $MyD88^{-/-}$ or HLA-DM mice with C57BL/6 mice for wild-type controls. Macrophages were derived from femur marrow cells cultured in bacterial grade dishes for 7–10 days in 25% LADMAC cell-conditioned medium (containing M-CSF (26)). To produce dendritic cells (27, 28), femur marrow cells were resuspended for 10 min in 0.83% NH_4Cl to lyse erythrocytes; incubated for 1 h at 4°C with combined supernatants of B hybridomas GK1.5 (anti-CD4), 53-6.72 (anti-CD8), RA3-3A1/61 (anti-B220), and 34-5-3S (anti-$I\text{-}A^{b/d}$) (American Type Culture Collection, Manassas, VA); and resuspended for 1 h at 37°C in complement (Accurate Chemical & Scientific, Westbury, NY). Cells were resuspended in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 5% FCS, 50 mM 2-ME, 25 mM HEPES (Invitrogen Life Technologies), 20 μg/ml gentamicin (Life Technologies), and 4 ng/ml GM-CSF (R&D Systems, Minneapolis, MN). Cells were cultured in six-well plates (3×10^6 cells/well), and nonadherent cells were removed every 2 days by gentle swirling and replacement of half of the volume with fresh medium containing GM-CSF. Dendritic cells were harvested by pipetting on day 5, incubated with anti-murine CD11c microbeads (Miltenyi Biotec, Auburn, CA; 100 μl beads/4×10^7 cells) for 20 min at 4°C, resuspended in PBS with 0.5% BSA and 2 mM EDTA (4×10^7 cells/500 μl), isolated with a MACS MS column (Miltenyi Biotec), washed, and resuspended in standard medium.

HSPs, Abs, and reagents

E. coli DnaK (StressGen Biotechnologies, Victoria, Canada) was >90% pure by SDS-PAGE analysis, and MTB HSP70 (Lionex, Braunschweig, Germany) was >95% pure by SDS-PAGE analysis. We also prepared MTB HSP70 from *E. coli* BL-21 transformed with MTB HSP70 in pET-23 (Novagen, Madison, WI), obtained through the Tuberculosis Research Materials and Vaccine Testing Contract (Colorado State University, Fort Collins, CO), which drives expression of His-tagged MTB HSP70 (His tag on C terminus of MTB HSP70). *E. coli* were induced with isopropyl β-D-thiogalactoside for 4 h and lysed with BugBuster (Novagen). His-tagged MTB HSP70 was purified under native conditions with nickel columns (Qiagen, Valencia, CA). Similar results were obtained with MTB HSP70 (Lionex) and His-tagged MTB HSP70. Although LPS contamination was detected in HSP preparations with the E-TOXATE *Limulus* amebocyte lysate assay (Sigma-Aldrich, St. Louis, MO) with maximum experimental LPS concentrations of 0.22–1.1 μg/ml for *E. coli* DnaK and <0.14 μg/ml for MTB HSP70, control experiments without HSP, but with addition of LPS (from *E. coli* O127:B8; Difco, Detroit, MI) up to 1.5 μg/ml showed that LPS did not alter results or replicate HSP effects.

FITC-labeled 22-mer extended OVA (319–340) peptide AESLKISQAVHAAHAEINEAGR (FITC conjugation at N terminus, 77% pure by HPLC) from Biosynthesis (Lewisville, TX) was used for all experiments, except for those with HSP:peptide complexes bound to latex beads, which used FITC-labeled 37-mer extended OVA peptide GISSAESLKISQAVHAAHAEINEAGREVVGLLVLLKK (Biosynthesis; >86% pure by HPLC). FITC-labeled extended OVA peptide (0.04 ml at 1 mM in H_2O) was incubated with 0.4 ml of *E. coli* DnaK (StressGen Biotechnologies) or MTB HSP70 at 2 mg/ml in 40 mM Tris-HCl, pH 7.5, at 37°C with rotation for 1 h. Unbound peptide was removed using a Micron YM-10 or YM-30 centrifugal filter device (Millipore, Bedford, MA) three times for 20 min at 14,000 rpm with washes in 40 mM Tris-HCl. A negative control sample of 0.4 ml 40 mM Tris-HCl, pH 7.5, and 0.04 ml of uncomplexed peptide (1 mM) was processed to insure that unbound peptide was removed. The HSP:FITC-peptide solution was analyzed with a Spectra Fluor Plus plate fluorometer (Tecan, Research Triangle Park, NC) to determine HSP-bound peptide concentration. DnaK and MTB HSP70 bound similar amounts of peptide with ~0.025 mol of peptide bound per mol of HSP (2.5% loading). In comparison, studies with eukaryotic HSPs reported 1–5% loading with peptide sequences from model Ag (17, 29) and 20% loading with Ag peptide containing sequences known to promote HSP binding (6). To make latex bead-HSP:peptide, protein G-Fluoresbrite YG carboxylate microspheres (one micron diameter; Polysciences, Warrington, PA) were incubated overnight at 4°C with anti-MTB HSP71 Ab (Stressgen Biotechnologies), which recognizes MTB HSP70. The microspheres were then washed, incubated with MTB HSP70:peptide for 2 h at 4°C, and washed with PBS.

Ag processing and presentation assays

Macrophages were detached with trypsin-versene (BioWhittaker), plated in 96-well flat-bottom plates at 10^5 cells/well, and incubated with 2 ng/ml rIFN-γ (Genzyme, Cambridge, MA) for 48 h. Cells were incubated with HSP:peptide (for 45 min, unless otherwise stated), fixed with 0.5% paraformaldehyde, washed, and incubated for 24 h with DOBW T hybridoma cells (10^5 cells/well) (30), which recognize OVA (323–339) presented by $I\text{-}A^d$ or $I\text{-}A^b$. Supernatants (100 μl) were frozen, thawed, and assessed for IL-2 using a colorimetric CTLL-2 bioassay (27, 31). Dendritic cells were subjected to the same protocol, except that plates were spun at 1800 rpm for 5 min between each wash step. Inhibitors used to probe Ag-processing mechanisms included 2-deoxy-D-glucose, chloroquine, and sodium azide (all from Sigma-Aldrich). The mAb to CD91 (5A6) was from PROGEN Biotechnik (Heidelberg, Germany), and isotype control IgG1 was from Zymed Laboratories (San Francisco, CA). Latex-OVA beads were made by noncovalent conjugation of chicken egg OVA (Sigma-Aldrich; A5503) to 2-μm latex beads (Polysciences).

Flow cytometry

Macrophages were incubated in 24-well plates (6.7×10^5 cells/well) for 48 h at 37°C with 2 ng/ml IFN-γ. Hsp:FITC-labeled peptide complexes were added for 25 min. Cells were then washed in PBS, detached by scraping, fixed in 1% paraformaldehyde, and analyzed by flow cytometry.

Results

E. coli *DnaK and MTB HSP70 promote processing and MHC-II presentation of chaperoned extended OVA peptide containing the OVA (323–339) epitope*

Exogenous mammalian and bacterial HSPs have been shown to deliver antigenic peptides to APCs and promote MHC-I processing and presentation of HSP-chaperoned peptides, generating $CD8^+$ T cell responses. We propose that exogenous HSPs may also deliver peptides for MHC-II processing and presentation to generate $CD4^+$ T cell responses. Because bacterial HSPs are associated with peptides derived from bacterial proteins, including bacterial Ags, bacterial HSPs released during phagolysosomal processing of phagocytosed bacteria could deliver bacterial Ags for MHC-II processing and presentation, contributing to antibacterial $CD4^+$ T cell responses. MHC-II Ag processing of HSP-chaperoned peptides has not been studied with either eukaryotic or bacterial HSPs. Our studies were designed to test the hypothesis that bacterial HSPs deliver exogenous peptides for MHC-II processing and presentation of constituent epitopes.

We tested whether an *E. coli* HSP, DnaK, could deliver a 22-mer extended OVA peptide (AESLKISQAVHAAHAEINEAGR, OVA (319–340)) for MHC-II processing and presentation of a constituent epitope (OVA (323–339)) by MHC-II. Extended OVA peptides have been used to study MHC-I processing and presentation of HSP-chaperoned peptide (8, 16, 18, 29, 32), and we

adapted this strategy for studies of MHC-II presentation of HSP-chaperoned peptide. APCs were incubated for 45 min with uncomplexed extended OVA peptide or *E. coli* DnaK:extended OVA peptide complexes. The cells were then fixed and incubated with DOBW T hybridoma cells to detect OVA (323–339):MHC-II complexes. Association of extended OVA peptide with *E. coli* DnaK significantly enhanced presentation of the OVA (323–339) epitope from the extended peptide by both macrophages (Fig. 1*A*) and dendritic cells (Fig. 1*B*). We conclude that *E. coli* DnaK can efficiently deliver chaperoned peptide for MHC-II presentation of constituent epitopes.

Additional studies were performed with MTB HSP70 to determine whether the above findings were idiosyncratic to *E. coli* DnaK or reflected properties shared broadly among bacterial HSPs. APCs were incubated with MTB HSP70:extended OVA peptide complexes for 60 min (Fig. 1*C*) or 45 min (Fig. 1*D*), fixed, and incubated with DOBW T hybridoma cells to detect OVA (323–339):MHC-II complexes. MTB HSP70 significantly enhanced MHC-II peptide presentation in both macrophages (Fig. 1*C*) and dendritic cells (Fig. 1*D*). The greatest relative enhancement of peptide presentation by MTB HSP with our readout assay was observed from 0.5–3 h of processing (Fig. 1*E*); plateau signal (maximum response of our T cell assay) was achieved within this time frame at typical concentrations of MTB HSP70:peptide, whereas presentation of uncomplexed extended OVA remained low from 0.5–3 h, but increased at longer times (beyond 3 h it is likely that MTB HSP70:peptide continued to produce peptide: MHC-II complexes that were not evident due to signal plateau). Thus, bacterial HSPs from both *E. coli* and MTB can deliver extended peptide for enhanced MHC-II Ag presentation of a constituent epitope.

Enhancement of MHC-II peptide presentation by bacterial HSP occurs only if peptide is complexed to the HSP and is not due to signaling initiated by uncomplexed HSP

Although HSP contributions to MHC-II Ag processing and presentation have not been examined, some immune functions have been attributed to bacterial HSPs. MTB HSP70 and chlamydial HSP60 stimulate cytokine secretion through CD40 and TLR4, respectively (33, 34). These observations suggested that modulation of MHC-II Ag processing by bacterial HSPs could be explained by HSP signaling to produce generalized enhancement of macrophage MHC-II Ag processing and presentation, regardless of whether the presented peptides were directly chaperoned by the HSP.

To distinguish generalized effects of HSP signaling from enhanced processing specific to HSP-chaperoned peptide, we explored the requirement for binding of extended peptide to HSP. Macrophages were incubated for 45 min with HSP-complexed extended OVA peptide or equivalent concentrations of uncomplexed extended OVA peptide plus MTB HSP70. APCs were fixed and assessed for presentation of OVA (323–339):MHC-II complexes. The presence of MTB HSP70 did not enhance processing and presentation of uncomplexed extended OVA peptide (Fig. 2*A*). OVA (323–339) presentation was enhanced only if exogenous extended peptide was complexed to the HSP.

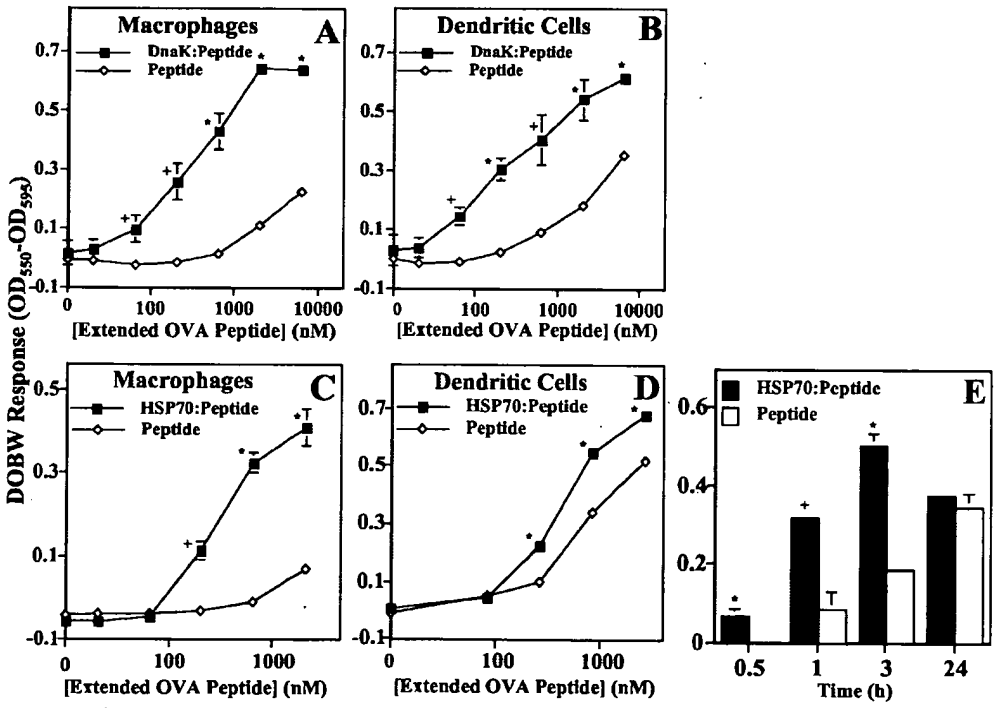

FIGURE 1. *E. coli* DnaK and MTB HSP70 enhance MHC-II Ag processing in macrophages and dendritic cells. Cells were incubated for 45 min (*A*, *B*, and *D*) or 60 min (*C*) with FITC-labeled extended OVA peptide complexed to *E. coli* DnaK or MTB HSP70. The cells were then fixed and incubated with OVA-specific MHC-II-restricted DOBW T hybridoma cells. Supernatants were assessed for IL-2 using a colorimetric CTLL-2 bioassay. *A* and *B*, *E. coli* DnaK enhances processing and presentation of extended OVA peptide by bone marrow-derived macrophages (*A*) and dendritic cells (*B*). *C* and *D*, MTB HSP70 enhances processing and presentation of extended OVA peptide by macrophages (*C*) and dendritic cells (*D*). *E*, MTB HSP70 produces rapid enhancement of extended OVA peptide processing by macrophages. Macrophages were incubated with 2.19 μM extended OVA peptide complexed to MTB HSP70 for the indicated periods. In this and other figures, the *x*-axes in panels showing dose-response experiments represent the concentration of extended OVA peptide, whether bound to HSP or uncomplexed. The results in each panel are representative of at least three independent experiments. Data points represent means of triplicate samples with SD. When error bars are not visible, they are smaller than the symbol width. Values of p resulting from a two-tailed t test comparing results with and without HSP are shown (+, $p < 0.05$; *, $p < 0.01$).

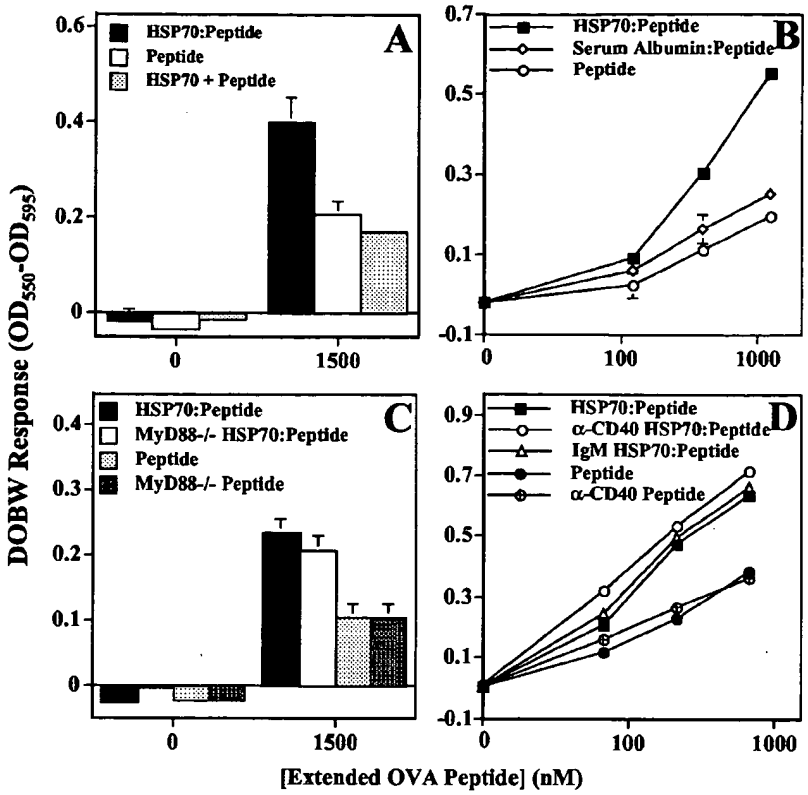

FIGURE 2. MTB HSP70 specifically enhances processing and MHC-II presentation of chaperoned peptide. After incubation with bacterial HSPs and extended OVA peptide, macrophages were assessed for OVA peptide presentation, as in Fig. 1. *A*, Macrophages were incubated for 45 min with MTB HSP70 complexed to extended OVA peptide (HSP70:Peptide) or uncomplexed extended OVA peptide alone. Alternatively, macrophages were incubated for 45 min with uncomplexed extended OVA peptide and MTB HSP70 (HSP70 + Peptide). *B*, Macrophages were incubated for 45 min with extended OVA peptide complexed to MTB HSP70, extended OVA peptide complexed to BSA, or uncomplexed extended OVA peptide. *C*, Macrophages from MyD88$^{-/-}$ and wild-type C57BL/6 mice were incubated for 45 min with extended OVA peptide, either alone or complexed with MTB HSP70. *D*, Macrophages were preincubated with blocking mAb to CD40 (α-CD40; 50 μg/ml) or isotype control Armenian hamster IgM (IgM; 50 μg/ml) for 30 min before and during a 45-min incubation with HSP:extended OVA peptide complexes. Presentation of extended OVA peptide without HSP was the same in the presence of either isotype control Ab or CD40-blocking Ab (data not shown due to overlapping symbols). The results in each panel are representative of at least three independent experiments. Data points represent means of triplicate samples with SD. When error bars are not visible, they are smaller than the symbol width.

To determine whether enhanced MHC-II processing and presentation of chaperoned peptide were related to specific HSP properties, we compared MHC-II presentation of OVA (323–339) after exposure of macrophages to extended OVA peptide bound to HSP or BSA (BSA bound peptide, as reported previously (29)). APCs were incubated with MTB HSP70:extended OVA peptide complexes or BSA:extended OVA peptide complexes for 45 min, fixed, and incubated with DOBW T hybridoma cells to detect OVA (323–339):MHC-II complexes. MTB HSP70 significantly enhanced MHC-II peptide presentation, but BSA did not enhance MHC-II peptide presentation in macrophages (Fig. 2*B*). Thus, HSP enhancement of Ag presentation was related to the ability of HSPs to promote processing or presentation of chaperoned peptide.

We performed experiments to determine whether TLR- and MyD88-dependent signaling by HSPs or potential microbial contaminants of bacterial HSP preparations (e.g., LPS) altered APC function to cause HSP enhancement of peptide presentation. LPS was excluded as a significant factor in our experiments, because LPS (up to 1.5 μg/ml, higher than maximal levels of LPS from HSP preparations) did not affect the processing of extended OVA peptide (data not shown). In addition, enhancement of MHC-II peptide presentation by MTB HSP70 was identical with wild-type and MyD88$^{-/-}$ macrophages (Fig. 2*C*). Finally, enhancement of MHC-II peptide presentation by MTB HSP70 was not affected by anti-CD40-blocking Ab (Fig. 2*D*). Although HSPs have been reported to signal through TLRs or CD40 to stimulate proinflammatory cytokines (33–37), our data indicate that enhancement of peptide presentation was not the result of signaling by HSPs or bacterial contaminants to alter overall processing functions of APCs. Furthermore, HSP-enhanced processing was specific to the HSP-chaperoned extended peptide that was delivered for processing and MHC-II presentation.

Active processing by APCs is necessary for enhanced presentation of HSP-chaperoned extended peptide

We tested whether bacterial HSP-chaperoned peptides required active processing, e.g., through endocytosis by viable APCs, or could be delivered directly to cell surface MHC-II molecules. To distinguish active processing from cell surface events, uptake and processing functions were inhibited by fixation or metabolic inhibition of APCs. Fixation of macrophages before addition of extended OVA peptide prevented enhancement of MHC-II peptide presentation by MTB HSP70 (Fig. 3*A*). Experiments with dendritic cells produced similar results; prefixation of dendritic cells prevented enhancement of MHC-II peptide presentation by MTB HSP70 (data not shown). Alternatively, macrophages were exposed to sodium azide and 2-deoxy-D-glucose for 90 min to deplete ATP and subsequently incubated with MTB HSP70:extended OVA peptide

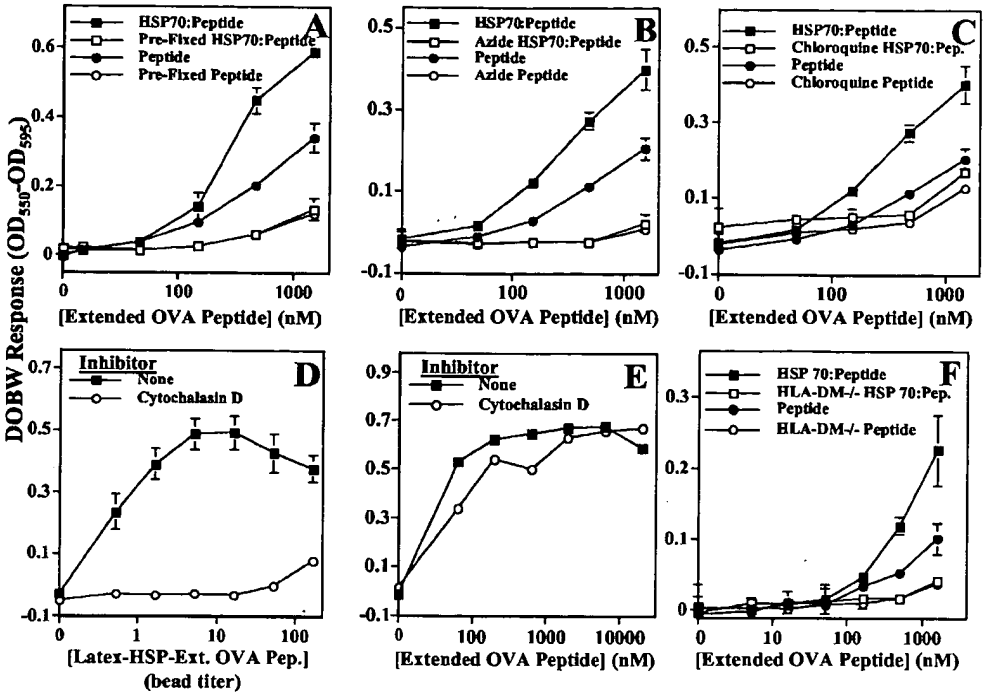

FIGURE 3. Active processing by APCs is required for MTB HSP70 to enhance MHC-II peptide presentation. Macrophages were exposed to inhibitors before and during incubation with MTB HSP70:extended OVA peptide complexes for 45 min. The cells were then fixed and assessed for presentation of OVA (323–339):MHC-II complexes, as in Fig. 1. *A*, Macrophages were fixed with 0.5% paraformaldehyde before incubation with MTB HSP70:peptide complexes. *B*, Macrophages were exposed to 30 mM sodium azide and 5 mM deoxy-D-glucose for 90 min before and during incubation with MTB HSP70:peptide complexes. *C*, Macrophages were exposed to 0.1 mM chloroquine for 30 min before and during incubation with MTB HSP70:peptide complexes. *D*, Macrophages were exposed to 10 μg/ml cytochalasin D for 15 min before and during a 30-min incubation with MTB HSP70:peptide complexes linked to protein G-coated latex beads by anti-MTB HSP70 Ab. Macrophages were then washed and incubated for 90 min with or without cytochalasin D. *E*, Macrophages were exposed to 10 μg/ml cytochalasin D for 15 min before and during incubation with soluble extended OVA peptide and processed as in *D*. *F*, Macrophages from HLA-$DM^{-/-}$ and wild-type C57BL/6 mice were incubated for 45 min with extended OVA peptide, either alone or complexed with MTB HSP70. The results in each panel are representative of three independent experiments. Data points represent means of triplicate samples with SD. When error bars are not visible, they are smaller than the symbol width.

complexes for 45 min. This metabolic inhibition blocked enhancement of extended OVA peptide processing and presentation by MTB HSP70 (Fig. 3*B*). Macrophages were also exposed to chloroquine for 30 min to neutralize the pH of vacuolar compartments and subsequently incubated with MTB HSP70:extended OVA peptide complexes for 45 min. Chloroquine also blocked enhancement of extended OVA peptide processing and presentation by MTB HSP70 (Fig. 3*C*). Presentation of uncomplexed peptide was reduced by fixation or metabolic inhibition (Fig. 3, *A–C*), possibly reflecting loss of active peptide uptake, but sufficient signal remained to determine that HSP enhancement did not occur under these conditions. Thus, active APC function or processing was required for HSP enhancement of peptide presentation.

To confirm that bacterial HSPs enhance Ag processing through an active process involving cellular uptake, we attached HSP:peptide complexes to latex beads and used cytochalasin D to inhibit actin-dependent phagocytosis of these beads (Fig. 3*D*). Cytochalasin D inhibits actin-dependent phagocytosis of particles such as latex beads, but does not inhibit endocytosis of soluble proteins, which is not dependent on actin microfilament function. Thus, to make uptake of HSP70:peptide sensitive to cytochalasin D, we conjugated it to 1-μm diameter latex beads. Macrophages were incubated with or without cytochalasin D for 15 min before and during incubation with bead-conjugated MTB HSP70:extended OVA peptide. Cytochalasin D significantly inhibited processing of MTB HSP70:extended OVA peptide beads (Fig. 3*D*). Control samples showed that cytochalasin D completely blocked phagocytic alternate MHC-I processing of whole OVA protein conjugated to latex beads (data not shown), as previously observed (38, 39). Presentation of uncomplexed extended OVA peptide was unaltered by cytochalasin D, indicating that cytochalasin D did not cause general loss of macrophage peptide presentation function (Fig. 3*E*). This experiment confirms and extends conclusions from experiments shown in Fig. 3, *A–C*, using an independent approach. Because bacterial HSP enhancement of extended OVA peptide processing and presentation is abolished in macrophages that are unable to internalize or actively process HSP:peptide complexes, we conclude that MTB HSP70 peptide complexes must enter the acidic environment of intracellular vacuolar compartments to access MHC-II Ag processing mechanisms to which HSP-chaperoned Ag can contribute.

To examine the molecular requirements for efficient processing of bacterial HSP:peptide complexes, HLA-$DM^{-/-}$ macrophages were used. HLA-DM is expressed inside endosomal compartments and is required for removal of class II-associated invariant chain peptide and subsequent loading of MHC-II molecules with peptides. Enhancement of MHC-II peptide presentation by MTB HSP70 was abolished in macrophages that lacked HLA-DM (Fig. 3*F*).

Bacterial HSPs enhance uptake of Ag

Enhancement of MHC-II Ag processing by bacterial HSPs could be mediated by enhanced uptake of peptide Ag as well as enhanced intracellular processing mechanisms. To assess whether *E. coli* DnaK or MTB HSP70 enhance uptake of extended OVA peptide,

macrophages were incubated with FITC-labeled extended OVA peptide either uncomplexed or complexed with bacterial HSP. Flow cytometry was used to determine the level of FITC-peptide uptake. In negative control samples, APCs were incubated with metabolic inhibitors (azide and 2-deoxy-D-glucose) for 90 min before and during the peptide incubation to inhibit endocytosis. MTB HSP70:peptide complexes produced a strong signal for peptide uptake with mean fluorescence value (MFV) of 293, and metabolic inhibitors reduced uptake to ~62% of this level (MFV = 182) (Fig. 4). In contrast, uptake of extended OVA peptide without HSP was much lower (MFV = 109) and was not substantially affected by addition of metabolic inhibitors (MFV = 99). Similarly, *E. coli* DnaK:extended OVA peptide promoted peptide uptake that was reduced by metabolic inhibitors to baseline uptake level (data not shown). In addition, MTB HSP70 enhanced uptake of chaperoned peptides by dendritic cells, similar to the results seen with macrophages (data not shown). In summary, MTB HSP70 and *E. coli* DnaK enhanced uptake of chaperoned extended OVA peptide through a mechanism that was reduced by metabolic inhibitors. Thus, bacterial HSPs mediate enhanced delivery of peptide Ags to APCs, and this mechanism may contribute to enhancement of MHC-II Ag processing and presentation.

Bacterial HSPs facilitate delivery and binding of chaperoned peptide to MHC-II under acidic conditions that mimic the vacuolar environment

In addition to enhancement of peptide uptake, bacterial HSPs may have active roles inside APCs to deliver peptides to MHC-II for binding and presentation. Although bacterial HSPs failed to deliver peptides to surface MHC-II molecules at neutral pH (Fig. 3*A*), we tested whether they could enhance loading of MHC-II at acidic pH (5.0) similar to that found in vacuolar compartments. APCs were fixed with paraformaldehyde, resuspended in citrate buffered saline at either pH 5.0 or 7.4, incubated with MTB HSP70:extended OVA peptide complexes for 45 min, fixed, and incubated with DOBW T hybridoma cells to detect OVA peptide:MHC-II complexes. At pH 7.4, MTB HSP70 did not enhance formation of

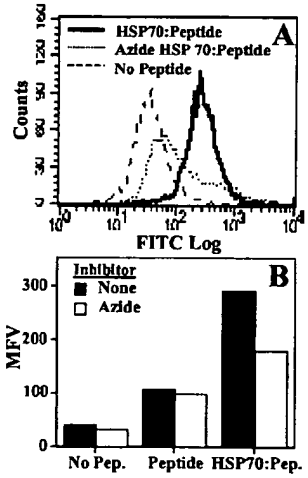

FIGURE 4. MTB HSP70 enhances uptake of chaperoned peptides. Macrophages were incubated with or without 30 mM sodium azide and 5 mM deoxy-D-glucose for 90 min before and during incubation for 25 min with FITC-labeled extended OVA peptide bound to HSP (6.96 μM final peptide concentration). The cells were then washed, fixed, and analyzed for uptake of FITC-labeled extended OVA peptide by flow cytometry. *A*, Representative histogram of MTB HSP70-enhanced uptake of extended OVA peptide. *B*, MFVs for MTB HSP70 enhancement of peptide uptake. Each panel is representative of three independent experiments.

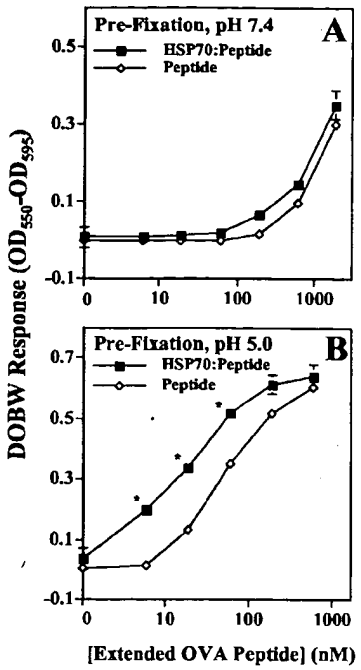

FIGURE 5. MTB HSP70 enhances MHC-II peptide presentation in acidic environments. Macrophages were fixed, incubated with MTB HSP70:extended OVA complexes in citrate buffered saline at pH 7.4 (*A*) or 5.0 (*B*) for 30 min, fixed again, and assessed for presentation of MHC-II: OVA complexes, as in Fig. 1. Data points represent means of triplicate samples with SD. The results in each panel are representative of at least three independent experiments. When error bars are not visible, they are smaller than the symbol width. Values of p resulting from a two-tailed t test comparing results with and without HSP are shown (*, $p < 0.01$).

OVA peptide:MHC-II complexes beyond the background level observed in the absence of MTB HSP70 (Fig. 5*A*). At pH 5.0, however, MTB HSP70 enhanced formation of OVA peptide:MHC-II complexes (Fig. 5*B*). These results indicate that bacterial HSPs can assist in MHC-II peptide loading in acidic environments. The binding of uncomplexed peptide was higher at pH 5.0 than pH 7.4 (Fig. 5, *A* vs *B*), consistent with previous observations that MHC-II molecules optimally bind or exchange peptides under acidic conditions (40, 41), but HSP-complexed peptide produced even greater binding of peptide to MHC-II. Thus, acidic vacuolar pH may promote release of HSP-chaperoned peptides and their binding to MHC-II molecules.

Discussion

HSPs enhance alternate MHC-I Ag processing of chaperoned self, tumor, or viral peptides (reflecting the range of proteins synthesized by mammalian cells) to promote MHC-I cross presentation and $CD8^+$ T cell responses (2, 4–6, 11, 13, 15, 16). It has also been demonstrated that overexpression of endogenous mammalian HSPs can enhance MHC-II presentation to $CD4^+$ T cells (19–22), although specific Ag-processing functions to which HSPs may contribute have not been addressed. Furthermore, MHC-II processing of exogenous HSP:complexes, either mammalian or bacterial, has not been studied. In particular, there have been no studies to test whether bacterial HSPs, which are naturally associated with bacterial peptides, deliver chaperoned peptides for MHC-II Ag processing and presentation.

The microbial peptides that are chaperoned by bacterial HSPs are a potentially important source of Ag to promote antimicrobial immunity. We propose that microbicidal mechanisms cause release of bacterial HSPs from phagocytosed bacteria, potentially

delivering antigenic HSP-chaperoned bacterial peptides into the phagolysosomal environment for processing and binding to MHC-II. In addition, bacterial HSPs released into the extracellular space (secondary to lysis of bacteria by complement or other mechanisms) may be internalized by APCs, allowing MHC-II processing of HSP-chaperoned bacterial Ags. In this study, we demonstrate that exogenous bacterial HSPs (*E. coli* DnaK and MTB HSP70) enhance uptake and MHC-II Ag processing of a chaperoned model peptide.

Some HSPs may stimulate signaling (e.g., chlamydial HSP60 signaling via TLR 4 (34) and MTB HSP70 signaling via CD40 (33, 37)) to increase expression of accessory factors (e.g., cytokines or costimulators), which may be important in vivo for generating responses by primary T cells. Our data establish a different mechanism whereby bacterial HSPs contribute directly to generation of peptide:MHC-II complexes from HSP-chaperoned peptide Ag (as detected by T hybridoma cells, which are less dependent on costimulatory molecules). This mechanism requires that peptides be complexed to the HSP and is independent of MyD88 and CD40. Thus, this enhancement by HSPs does not involve a generalized increase in MHC-II Ag presentation, but specifically involves the delivery of HSP-chaperoned peptides for processing and presentation by MHC-II.

Two potential mechanisms that could explain enhancement of MHC-II Ag processing by bacterial HSPs are increased uptake and enhanced intracellular processing. Both of these mechanisms are supported by our observations. Uptake of peptide complexed to either *E. coli* DnaK or MTB HSP70 was significantly higher than uptake of uncomplexed peptide as measured by flow cytometry. In some systems, CD91 has been reported to be involved in uptake of exogenous HSP:peptide complexes for alternate MHC-I processing and presentation (10, 18, 42), but MHC-II processing of MTB HSP70:peptide complexes was not altered by the addition of anti-CD91-blocking Ab (data not shown). The reason for this difference is unknown, but intracellular trafficking of CD91 and differences in compartmentalization of MHC-II and alternate MHC-I processing may contribute. These observations coupled with the finding that bacterial HSP enhanced uptake of peptide in the present studies suggest that receptors other than CD91 may also be involved in uptake of HSPs.

In addition to assisting in uptake, bacterial HSPs may enhance intracellular processing of chaperoned Ag. Although bacterial HSPs did not enhance binding of peptide to MHC-II at neutral pH, HSP-enhanced binding was evident at pH 5.0. Thus, HSPs may release chaperoned peptides at the acidic pH of vacuolar compartments, making these peptides available for binding to MHC-II. We speculate that this could involve direct interactions between HSPs and MHC-II, leading to release of chaperoned peptide, but this hypothesis requires future experimental testing. It is interesting to note that MHC-II molecules optimally bind or exchange peptides under acidic conditions (40, 41). Thus, acidic vacuolar pH may promote both release of HSP-chaperoned peptides and their binding to MHC-II molecules.

We propose that bacterial HSPs deliver antigenic HSP-chaperoned peptide Ags that contribute to the generation of $CD4^+$ T cell responses during infection of mammalian hosts with bacterial pathogens. This mechanism could be particularly important during infection with bacterial pathogens for which $CD4^+$ T cell responses contribute to host immunity. APCs may encounter bacterial HSPs following phagocytosis and phagolysosomal degradation of bacteria, which may release bacterial HSPs directly into the phagosomal Ag-processing environment (where MHC-II molecules are present). Alternatively, bacterial HSPs that have been released in the extracellular space by microbicidal mechanisms (e.g., complement) may be internalized for subsequent intracellular processing and MHC-II presentation. In addition to physiological roles in processing of bacterial Ags, bacterial HSPs have the potential to contribute to vaccine efficacy. HSPs, including bacterial HSPs, could be incorporated in vaccines to stimulate $CD4^+$ T cell responses that are crucial to immune responses against bacteria.

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CD40-independent engagement of mammalian hsp70 by antigen-presenting cells.

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CD40 has been suggested previously to be a receptor for mammalian murine hsc73 (hsp70). We have examined, in vitro and in vivo, the role of CD40 in the interaction of murine dendritic cells and macrophages with hsp70, using several independent parameters including cell surface binding, translocation of NF-kappaB, stimulation of release of TNF-alpha, representation of hsp70-chaperoned peptides, and priming of CD8(+) T cells. The various consequences of hsp70-APC interaction were compared between CD40(+/+) and CD40(-/-) mice and were found to be identical in kinetics and magnitude. These data strongly indicate that all known effects of mammalian hsp70 on APCs are mediated in a CD40-independent manner. In light of the earlier demonstration that mycobacterial hsp70 binds murine CD40 and stimulates the APCs through it, our data indicate that CD40 can discriminate between self and mycobacterial hsp70 and is thus a receptor for patterns associated with microbial pathogens.

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